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Toxic effects of nickel alone and in combination with microplastics on early juveniles of the common goby (*Pomatoschistus microps*)

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Resumo

Os estuários estão entre os ecossistemas aquáticos mais produtivos. No entanto, estão sujeitos a elevados níveis de pressão antropogénica devido ao crescimento exponencial da população e aumento da industrialização. Os metais estão entre os contaminantes ambientais mais relevantes em ecossistemas estuarinos. O níquel, é um metal amplamente utilizado em várias actividades antropogénicas, especialmente a nível industrial. Por esse motivo, pode ser encontrado no ambiente em níveis superiores relativamente às concentrações decorrentes da sua ocorrência enquanto elemento natural. Tem sido encontrado na coluna de água, sedimentos e organismos em estuários da zona NW de Portugal. Os microplásticos são polímeros orgânicos sintéticos (< 5mm), produzidos a partir de recursos não renováveis. São considerados contaminantes ambientais emergentes de elevada relevância, especialmente em ecossistemas aquáticos, principalmente por poderem atuar como vetor de entrada de outros contaminantes ambientais de elevada toxicidade nas cadeias alimentares e pela capacidade de interagir com os efeitos de outros contaminantes no biota. Quer o níquel quer os microplásticos têm a capacidade de induzir efeitos tóxicos em organismos aquáticos, incluindo em peixes. O objetivo central deste estudo foi investigar os efeitos tóxicos do níquel e de microplásticos em juvenis precoces (grupo 0+) de *Pomatoschistus microps* de populações selvagens dos estuários dos rios Minho e Lima, quer após exposição individual a cada um dos agentes quer após exposição simultânea a ambos, relativamente à exposição a distintas condições ambientais durante as fases anteriores do desenvolvimento. Especificamente pretendeu-se testar as seguintes hipóteses: (i) juvenis de estuários com diferentes níveis de contaminação ambiental têm sensibilidades diferentes ao níquel; (ii) a presença de microplásticos na água influência a toxicidade do níquel para os juvenis.

O trabalho foi dividido em três fases. A primeira fase consistiu na aprendizagem das metodologias para capturar os juvenis em estuários, mantê-los em laboratório, realizar bioensaios com esta espécie e determinar diversos critérios de efeito, nomeadamente mortalidade, o desempenho predatório pós-exposição (doravante designada por performance predatória), níveis de peroxidação lipídica (LPO) e actividade das enzimas acetilcolinesterase (AChE), glutationa S-transferase (GST) e etoxiresorufina-O-deetilase (EROD). Na segunda

fase do trabalho foi efetuado um bioensaio para comparar os efeitos induzidos pelo níquel em juvenis provenientes dos estuários dos rios Minho e Lima, os quais têm diferentes níveis de contaminação ambiental, de forma a testar a primeira hipótese. Finalmente, foram avaliados os efeitos induzidos por exposição a diferentes concentrações de níquel na presença de microplásticos e por microplásticos isolados em juvenis de ambos os estuários, de forma a testar a segunda hipótese.

No primeiro bioensaio, o níquel induziu mortalidade em juvenis de ambos os estuários, tendo sido determinadas concentrações letais medianas (LC_{50}) de 46.721 mg/l para juvenis do estuário do rio Minho e de 44.025 mg/l para juvenis do estuário do rio Lima. Em juvenis de ambos os estuários, o níquel reduziu o desempenho predatório, induziu a atividade da GST e aumentou os LPO a concentrações iguais ou superiores a 12.5 mg/l, 25.0 mg/l e 25.0 mg/l, respetivamente. Estes resultados indicam que os juvenis dos estuários dos rios Minho e Lima têm sensibilidades semelhantes ao níquel, rejeitando-se a hipótese 1, o que significa que a exposição durante fases anteriores do desenvolvimento dos organismos não influenciou a sua sensibilidade ao níquel.

No segundo bioensaio, as LC_{50} do níquel na presença de microplásticos determinadas para juvenis dos estuários do Minho e do Lima foram 34.698 mg/l e 47.080 mg/l, respetivamente. Além disso, em juvenis de ambos os estuários expostos simultaneamente aos dois agentes, observou-se uma redução do desempenho predatório e indução da atividade da GST, a concentrações iguais ou superiores a 6.3 e 12.5 mg/l, respetivamente. Em juvenis do estuário do Minho observou-se ainda uma indução da atividade da AChE a concentrações iguais ou superiores a 3.1 mg/l. A comparação dos resultados dos ensaios com e sem microplásticos para cada estuário indicam que a presença de microplásticos na água influência a toxicidade do níquel para juvenis de *P. microps*, pelo que se aceita a segunda hipótese.

Os resultados obtidos demonstram que a exposição de peixes juvenis estuarinos ao níquel induz efeitos tóxicos, reduzindo a eficiência na captura de alimento, o que leva a um decréscimo da energia necessária para o desenvolvimento dos organismos e outras funções como locomoção e reprodução, afetando a população inteira. Sendo os peixes zooplanctívoros predadores intermediários

importantes, um desiquilíbrio entre comunidades pode ser causado, induzindo graves repercussões ao nível do ecossistema.

Além disso, os microplásticos demonstraram capacidade para interagir a nível toxicológico com o níquel, destacando a importância de efetuar mais estudos de modo a investigar os seus mecanismos de ação como poluente ambiental emergente.

Palavras-chave: *Pomatoschistus microps*, níquel, microplásticos, mortalidade, desempenho predatório, biomarcadores, interações toxicológicas.

Abstract

Estuaries are among the most productive aquatic ecosystems. However, due to the human population exponential growth and great increase in industrialization, a considerable number of these ecosystems are under great anthropogenic pressure. Metals are one of the most relevant environmental contaminants in estuaries. Nickel is a metal widely used in anthropogenic activities, especially industrial ones. Thus, it is commonly found in the environment at concentrations above background levels. In estuaries of the NW Portuguese coast, nickel was found in the water, sediments and organisms. Microplastics are synthetic organic polymers (< 5mm) made from nonrenewable resources considered emerging contaminants of high concern, especially in aquatic environments, mainly because of their capability to act as vector for the entry of other environmental contaminants of high toxicity into the food-webs, and their ability to interact with the effects of other pollutants on the biota. Both nickel and microplastics are able to induce toxic effects on estuarine organism, including fish. The main objective of this Thesis was to investigate the toxic effects of nickel and microplastics in early juveniles (0^+ group) of *Pomatoschistus microps* from wild populations of Minho and Lima river estuaries, after exposure to the agents individually and in combination, regarding previous distinct long-term exposure to different levels of environmental contamination in early development stages. Especially, it was intended to test the following hypotheses: (i) juveniles from estuaries with different levels of environmental contamination have different sensibilities to nickel; (ii) the presence of microplastics in water influences the toxicity of nickel towards juveniles.

The work was divided in three phases. The first phase consisted of learning methodologies to capture juveniles in estuaries, their mainteinance in laboratory, performing bioassays with the species and determination of several effect criteria, including fish mortality, post-exposure predatory performance (hereafter designated as predatory performance), lipid peroxidation levels (LPO) and activities of the enzymes acetylcholinesterase (AChE), glutathione S- transferase (GST) and ethoxyresorufin-O-deethylase (EROD).

In the second phase of the work, was performed a bioassay to compare the effects of nickel induced in juveniles from populations from Minho and Lima river estuaries, which have different backgrounds of environmental contaminantion

levels, in order to test the first hypothesis. Finally, was performed a bioassay to evaluate the effects of exposure to different nickel concentrations combined with microplastics and microplastics alone induced in juveniles from both populations, in order to test the second hypothesis.

In the first bioassay, nickel induced mortality in juveniles from both estuaries, with determination of median lethal dose (LC_{50}) of 46.721 mg/l and 44.025 mg/l for fish from Minho and Lima river estuaries, respectively. In juveniles from both estuaries, nickel induced an inhibition in the predatory performance, an increase in GST activity and LPO levels in concentrations equal or above 12.5 mg/l, 25.0 mg/l and 25.0 mg/l, respectively. These results indicate that juveniles from Minho and Lima river estuaries have similar susceptibility to nickel, rejecting the first hypothesis, meaning that different exposures during earlier development stages of organisms do not influence the sensitivity to nickel.

In the second bioassay, nickel LC_{50} in the presence of microplastics were determined for juveniles from Minho and Lima river estuaries, with values of 34.698 mg/l and 47.080 mg/l, respectively. Also, juveniles of both estuaries exposed to nickel and microplastics combined shown a reduction in the predatory performance and an increase in GST activity, in concentrations equal or above 6.3 and 12.5 mg/l, respectively. Juveniles from Minho river estuary showed also an increase in AChE activity in concentrations equal or above 3.1 mg/l. Comparing the results from the two bioassays, with and without microplastics, from both estuaries, the presence of microplastics in water shows to influence the toxicity of nickel towards juveniles of *P. microps*, validating the second hypothesis.

The results from this estudy, indicates that exposure of juveniles of estuarine fish to nickel induces toxic effects, reducing the predation efficiency in the capability to chase and ingest food, leading to low levels of energy required to organisms development and other functions such as locomotion and reproduction, affecting the whole population. Zooplanctivoros fish have a key function as intermediate predator and so, serious consequences can occur at the ecosystem level.

Also, microplastics showed the capability to make toxicological relevant interactions with nickel, highlighting the importance of carryng out more studies to investigate their mechanisms of action as environmental emergent stressor.

Keywords: *Pomatoschistus microps*, nickel, microplastics, mortality, predatory performance, biomarkers, toxicological interactions.

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List of Abbreviations

AChE - Acetylcholinesterase

ASW – Artificial saltwater

BHT - Butylated hydroxytoluene

CAT - Catalase

CAS – Chemical Abstracts Service

CDNB - 1-chloro-2,4-dinitrobenzene

ChE - Cholinesterases

CYP1A - Cytochrome P450

DI – Deionized

DNA – Deoxyribonucleic acid

DTNB - 5-5'-dithio-bis-(2-nitrobenzoic acid)

DTT - Dithiothreitol

EC_{50} - Half maximal effective concentration

ERA - Ecological or environment risk assessment

EROD - Ethoxyresorufin-O-deethylase

F – Degrees of freedom

GSH – Glutathione (reduce form)

GST – Glutathione-*S*-transferase

LC_{50} – Median lethal dose

LPO – Lipid peroxidation

MP - Fluorescent polyethylene red microspheres

MSFD - Marine Strategy Framework Directive

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced form)

NAO - North Atlantic Oscillation

n.d. – No data

Ni – Nickel

NOAA - National Oceanic and Atmospheric Administration

NW – North-west

OECD – Organisation for economic co-operation and development

P - Significance

PAH – Polycyclic aromatic hydrocarbons

PCB - Polychlorinated biphenyl

PChE – Pseudo cholinesterases

POP – Persistant organic pollutant

ROS – reactive oxygen species

SD – Standard deviation

SEM – Standard error of the mean

SOD – Superoxide dismutase

SST – Sea surface temperature

TBA - Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substances

TCA - Trichloroacetic acid

Tris-HCL - Tris(hydroxymetil)-aminomethane

u.p. – Ultra-pure

UK – United Kingdom

USA – United States of America

WFD – Water Framework Directive

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1. Introduction

1.1. Ecotoxicology

Ecotoxicology, a term first mentioned in 1969 by René Truhaut (*Committee of the International Council of Scientific Unions*, Stockholm) (Truhaut, 1977), is a recent wide scope scientific field that aims to understand, predict and prevent the adverse effects of chemical substances (natural or synthetic), considered environmental contaminants, and other stressors *(e.g.* temperature, salinity) in the environment from biota, population and community to ecosystem (Magalhães & Filho, 2008; Beketov & Liess, 2012). It combines the principles of both Toxicology and Ecology, *i.e.* uses the study of the identification of nature, mechanism and toxicities of chemicals and their potential harmful effects on organisms (Williams *et al.*, 2000), applied not only to them but to their interactions with the environment at all levels of biological organization in the ecosystem, under realistic exposure conditions (Chapman, 2002). In an ecotoxicology approach, it's used monitoring and risk assessment programs, laboratory bioassays with lethal dose and effect concentration of chemicals establishment, behavioural assessment of organisms and biomarkers as effect criteria, allowing the evaluation of contaminants separately or in combination, in specific organisms and conditions. The assessment of this environmental impact is important not only to the ecosystems heath, but also to the human health.

1.2. Estuaries

According to Pritchard (1967), an estuary is a partly enclosed coastal body with a free connection to the open sea, forming a transition zone between fluvial and marine environments. The mix of both influences (flows of freshwater, tides and waves with influx of saline waters) provides high levels of nutrients, making estuaries among the most biologically productive natural habitats (Jackson, 2013; Monteiro *et al.*, 2007; Serafim *et al.*, 2012), establishing relevant ecological relations with other environments (Nyitrai *et al.*, 2013). With food, important essential habitats, which provides protection, migratory routes, nursery and reproduction grounds, estuaries have high biodiversity and therefore are considered not only genetically but also ecologically valuable and very important

to the economic level (Dolbeth *et al*., 2008; Monteiro *et al*., 2007, 2006; Nyitrai *et al*., 2013; Serafim *et al*., 2012). Nevertheless, with settlement and high exploitation by the human population, estuaries are subjected to great anthropogenic pressures (especially industrialization), leading to significant changes to these ecosystems and/or considerable environmental impacts (Azevedo *et al*., 2013; Berrebi *et al*., 2005; Monteiro *et al*., 2007; Serafim *et al*., 2012). As consequence, the presence of contaminants of several origins is, most of the times, increasingly high and therefore estuaries are impacted systems often considered sinks of pollution (Almeida *et al*., 2011).

1.3. Nickel

A significant class of estuarine contaminants is the group of metals, especially due to inputs from upstream catchments close to metropolitan areas and industries (Almeida *et al*., 2011; Vieira *et al*., 2009). According to Vieira *et al*. (2009), metals have the ability to cause biochemical and physiological damage in aquatic organisms, including fish.

Nickel, hereafter indicated as Ni, is a silvery-white color malleable transition heavy metal, hard at room temperature, corrosion resistant and with good capacity to conduct electricity and heat (Williams *et al*., 2000; Casarett & Klaassen, 2008). Ni is naturally present in nature at low levels, making part of the metallic elements of the earth's crust (Denkhaus & Salnikow, 2002; Williams *et al*., 2000). It occurs in the atmosphere by soil dust, sea salt, volcanoes, forest fires, vegetation exudates, and in aquatic ecosystems by erosion/degradation of rocks and soils, run offs and atmospheric deposition (Eisler, 1998). Making human population exposed to low levels of Ni everyday through water, air and food, generally with no toxicological concerns (Casarett & Klaassen, 2008). Widely used in industry, higher levels of Ni can enter the biosphere, especially in aquatic environments, by anthropogenic activities/sources: mining, smelting, plating, refining, alloy processing, coin minting, scrap metal reprocessing, electronic electroplating, batteries, fossil fuel combustion, waste incineration, milling, refinery-Ni production, and others, (Eisler, 1998; Jiang *et al*., 2013; Casarett & Klassen, 2008; Kubrak *et al*., 2012). Although considered a micronutrient (Pane *et al*., 2003) or an essential trace element for several groups of organisms, mainly

plants, microorganisms and invertebrates (Eisler, 1998; Gikas, 2008; Jiang, *et al*., 2013; Pourkhabbaz *et al*., 2011), Ni shown to become toxic when in elevated concentrations (Jiang *et al*., 2013). Ni typical levels in the environment are <100 ng/m^3 in air, among 4-80 mg/kg in soil, about 2µg/l in drinking water (Williams *et al*., 2000), and between 1 to 75 µg/l in estuaries, rivers and lakes (Eisler, 1998). In contaminated fresh waters, Ni concentrations can reach to 2500 µg/l (Eisler, 1998) and exceptional maximums of *e.g.* 183000 µg/l near a Ni refinery in Ontario (Chau & Kulikovsky-Cordeiro, 1995). Once entered into the environment, Ni does not suffer degradation, only valence transformations and transportation between fractions (Pourkhabbaz *et al*., 2011; Lushchak, 2008). In the aquatic ecosystems, the oxidation state Ni^{2+} is the most prevalent Ni form, which has great affinity to interact with organic and inorganic compounds (Casarett & Klassen, 2008; Svecevicius, 2010). Some of the Ni species are considered extremely toxic with immunotoxic, mutagenic and carcinogenic properties (Jiang *et al*., 2013; Kubrak *et al*., 2012, 2013; Pourkhabbaz *et al*., 2011). Among them, Ni carbonyl and Ni sulfides, are considered the most dangerous, both with carcinogen characteristics (Casarett & Klassen, 2008; William *et al*., 2000; Eisler, 1998). Also, the United States Environmental Protection Agency (USEPA) has classified the Ni refinery dust (Ni carbonyl mainly) and Ni sulfides as class A carcinogens (Williams *et al.,* 2000). Adverse effects of Ni that have been documented in humans, goes from relatively nontoxic exposures by "contact" absorption causing skin dermatitis, to exposures by inhalation resulting at severe lung and nasal sinus cancers (Eisler, 1998; Williams *et al*., 2000). Ni carbonyl poisoning can be extremely toxic, at first causing headache, nausea, cough and vomiting, to pneumonia, respiratory failure, cerebral edema and death. Exposure to Ni sulfides, mainly in nickel processing industry workers, shown to induce respiratory tract cancer (Eisler, 1998).

Concerning fish, according to Eisler (1998) review, the action mode of Ni toxicity and carcinogenic effects are related to the oxidative damage of DNA and proteins and to inhibition of cellular antioxidant defenses, by accumulation of Ni ions within cells and generating oxidative radicals (Williams *et al*., 2000). Fish experience visible signs of Ni intoxication when start to swim to surface, have rapid opercular and mouth movements, convulsions and loss of equilibrium. Associated with the respiratory system (Pane *et al*., 2003), the toxicity effects known can be destruction of gill lamellae due to decreased ventilation rate,

contractions of vascular smooth muscle, blood hypoxia, decrease in glycogen levels in liver and muscle with increase blood lactic acid and glucose levels, reduction of superoxide dismutase (SOD) and depressed hydrogen peroxide production and even death (see review Eisler, 1998).

1.4. Microplastics

Plastics are synthetic long chains of polymers produced from organic and inorganic raw material extracted from oil, coal and natural gas, such as carbon, silicon, hydrogen, nitrogen, oxygen and chloride (Cole *et al*., 2011; Ivar do Sul & Costa, 2014; Shah *et al*., 2008). The most commonly used polymers, and therefore found in the environment, are polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyethylene terephthalate (PET) and polyethylene (PE) (Ivar do Sul & Costa, 2014). Versatile, resistant, light weight, with low electric and thermal conduction and bio-inertness, allied to the easy low cost production (Andrady, 2011; Frias *et al*., 2010; Sivan, 2011), makes them suitable to unlimited applications, since industry (*e.g*. electronics, automotive, construction), agriculture, sports, health, the most important, packaging (PlasticsEurope, 2013; Shah *et al*., 2008), to daily basis products like toothbrushes and facial cleansers (Fendall & Sewell, 2009), among many others, substituting the usual resources of paper, glass, metal (Andrady, 2011), wood and leather (Sivan, 2011). Extremely practical, plastic has become essential part of the modern lifestyle and economy (PlasticsEurope, 2013), with an increasing production of more than two million tons of plastic globally produced per year from middle of the last century (Andrady, 2011), reaching the 288 million tons in 2012 (PlasticsEurope, 2013). Demographic changes towards the coastal areas, extensive use of the ocean (*e.g*. fishing, recreational and maritime activities) (Andrady, 2011) associated to this huge demand and the extremely low rate of plastic degradation (decades to centuries to degrade completely) (Cole *et al*., 2014) has, as a consequence, their uncontrollable accumulation in the environment, mainly in marine ecosystem (Sanchez *et al*., 2014). First reported by Carpenter and Smith in North Atlantic Ocean in 1972 (Ivar do Sul & Costa, 2014), nowadays, plastic items are the main component of marine debris (60 to 80%) (Lusher *et al*., 2013; Setälä *et al*., 2014), with fishing gear, ropes and nets (24%), fragments (20%), and packaging (17%) as

the main components (GEF, 2012) widely spread all across the world's oceans and seas (Collignon *et al*., 2013). Large plastic debris or macroplastics, have been widely investigated and revealed environmental issues (Cole *et al*., 2011) with several important physical impacts on marine organisms (Martins & Sobral, 2011). Entanglement, ingestion (mistaken for food) and death by physical injuries, starvation or suffocation are well documented in mammals, reptiles (Cole *et al*., 2011), cetaceans, sea turtles (Andrady, 2011), sea birds (Moore, 2008), and recently fish (Fossi *et al*., 2012). In the environment, these macroplastics are subject to degradation processes with reduction of molecular weight of the polymer, becoming weak and breaking up in increasingly smaller fragments through time (Andrady, 2011; Claessens *et al*., 2013). Particles with less than 5 mm were defined as microplastics by the National Oceanic and Atmospheric Administration (NOAA) (Moore, 2008). Microplastics resulting from this fragmentation are defined as secondary origin (Ivar do Sul *et al*., 2013), the main source of microplastics to the environment (Andrady, 2011). Due to the continuous weathering of plastic debris, microplastics concentration tends to be several orders of magnitude higher than macroplastics (Wright *et al*., 2013), mainly in land (*e.g*. beach) where initial degradation processes are much faster. Microplastics can be also of primary origin, purposely developed to have less than 5 mm, generally used in cosmetics (*e.g*. exfoliating), air-blasting technology (remove rust and paint usually in engines and boat hulls) and recently as drug vectors in medicine, with direct introduction in the environment by runoff discharges (Cole *et al*., 2011). In marine environment and recently identified in freshwater habitats (Cole *et al*., 2014), microplastics can be found all across the world (Lusher *et al*., 2013), through surface, water column and within benthos (Collignon *et al*. 2013), from shorelines far to open sea (Depledge *et al*., 2013; Lusher *et al*., 2013) and abyssal depths far away from human influence (Fendall & Sewell, 2009). Convergent regions and gyres (*e.g.* The Kuroshio Current, North Pacific Gyre) (Fendall & Sewell, 2009), are the locations where microplastics have tendency to accumulate (Collignon *et al*., 2013).

Ubiquitous in the environment and becoming available to smaller organisms, microplastics have become the focus of plastic pollution, with the status of emergent pollutant of major concern (Fossi *et al*., 2012), a class of contaminants newly discovered in the environment which impact and long term effects to both environment and human health are not yet well understood (OECD, 2012),

recently included in marine protection strategies like European Marine Strategy Framework Directive (MSFD) and NOAA Marine Debris Program (Cole *et al*., 2014). The main concern about microplastics it's their accessibility to an even larger range of organisms (Setälä *et al*., 2014). Given their size, they will be part of the same size range of sediments and plankton, and therefore, much likely to be mistaken by food by organisms (Sivan, 2011). Available to ingestion by low trophic biota, filter and deposit feeders, detritivores and planktivores it is not the possible physical harm the adverse effect in focus right now, but specially their capability to act like harmful substances vector (Fendall & Sewell, 2009; Wright *et al*., 2013), representing a route to persistent bioaccumulative toxic substances to enter and be transferred along the trophic food web (Andrady, 2011; Fossi *et al*., 2012; Setälä *et al*., 2014). The potential toxicity is associated to the additives and monomers they originally have (Setälä *et al*., 2014) (phthalate, bisphenol A, PE, PP, polycyclic aromatic hydrocarbons (PAH's)), and the pollutants present in the environment (dithiothreitol (DTT), polychlorinated biphenyl's (PCB's), (PAH's (Fossi *et al*., 2012), persistant organic pollutants (POP's) (Wright *et al*., 2013), heavy metals (Cole *et al*., 2011)), successfully absorb from water due to their relatively large area to volume ratio and hydrophobic affinity (Cole *et al*., 2014; Setälä *et al*., 2014), to leached out from the microplastic inside biota. This substances accumulate in microplatics in several orders of magnitude higher than in the water (*e.g.* with POP's are up to six orders of magnitude higher (Wright *et al*., 2013)), meaning that the "older" the microplastic, the more toxic it is (Fendall & Sewell, 2009). Regardless of the conception of no interaction between plastics and aqueous metals, microplastics metal adsorption is mentioned by several authors (Cole *et al*., 2011; Ivar do Sul & Costa, 2014; Oliveira *et al*., 2013). The exposure to this substances can interfere with biological processes causing potential toxic effects (*e.g*. endocrine disruption; carcinogenicity), and both bioaccumulation and biomagnification can occur up through the trophic levels of the food web (Wright *et al*., 2013). For example, microplastics found in seal scat, supposed to have *myctophid* fish as source, which feed on copepods of the same size of microplastics, which potentially already consumed microplastics also (Fendall & Sewell,2009), or crabs feed with tissue of mussels, previously exposed to microplastics in laboratory, showing evidence of bioaccumulation (Setälä *et al*., 2014). Ingested microplastics have been documented in gastrointestinal tracts of planktivorous fish in North Gyre (*Myctophidae, Stomiidae, Scomberesocidae*) (Fossi *et al.,* 2012) and in the English Channel (Lusher *et al*., 2013). In the base of

the food chain, zooplankton community have a key role on marine food web, with the responsibility of allowing to microplastics to pass through it, possibly reaching top predators, some of them commercially consumed, potentially jeopardizing human health (Cole *et al*., 2014; Fendall & Sewell, 2009; Wright *et al*., 2013**).** The presence of microplastics pollution is recognized in estuarine environments worldwide, however, very little is known about wildlife effects (Ivar do Sul & Costa, 2014; Sanchez *et al*., 2014). The concern about microplastics should be on their capability to modify entire population's structure, not only the effects on individual organisms (Wright *et al*., 2013). Even if the plastic production stops now, with the uncontrollable accumulation, low degradation rate and global dispersion, microplastics still will be a global increasing complex ecological problem, with effects on every kind of organisms, us included (Cole *et al*., 2014; Depledge *et al*., 2013; Fossi *et al*., 2012; Ivar do Sul *et al*., 2013; Martins & Sobral, 2011; Setälä *et al*., 2014; Sivan, 2011). About 660 marine species worldwide are recognized, in some way, to be affected by the plastic contamination (GEF, 2012).

1.5. Biomarkers

After several designations, biomarkers are commonly recognized as tools that attempt to make the link between individual biological responses and the potential harmful effects to the population due to external contamination sources that might be exposed (Van der Oost *et al*. 2003). Alterations in cellular, biochemical or physiological processes, measured in body fluids, cells or tissues and behavioral processes of the organisms that are considered beyond normal health variations demonstrating signals of environmental pollution (Peakall & Walker, 1994; Sanchez & Porcher, 2009; Van der Oost *et al*. 2003). Provide information about not only of the quantification of chemicals in the environment, but also about their bioavailability to biota (Van der Oost *et al*. 2003) and their mechanism of action (Sanchez & Porcher, 2009). Biomarkers give evidences of populations' health, acting as early-warning signals of environment risks (Payne *et al*., 1987), enabling to avoid extreme and/or irreversible damages on both environment and populations' structure (McCarthy & Shugart, 1990). Therefore, are considered very important tools in monitoring environment health status assessment programs (*e.g.* Ecological or environment risk assessment (ERA) (Van

der Oost *et al*. 2003) and implementation of Water frame directive programs (WFD) (Sanchez & Porcher, 2009)). Fish biomarkers are important due to the the link between aquatic food-webs of low and upper levels (Van der Oost *et al*. 2003). The relation between the biological responses and the chemical exposure may not be always easy to interpret, due to different characteristics between species, environment adaptability, interactions of pollutants in mixture (Sanchez & Porcher, 2009), or because these alterations do not necessary have negative effects in higher biological levels (Vieira *et al*., 2009). So, a better evaluation about the effects and toxicity of the contaminants and environmental quality will be provided by performing a set of biomarkers (Van der Oost *et al*. 2003). According to Van der Oost et al. (2003), biomarkers can be divided in several main groups, being the biotransformation, oxidative and physiological stress the ones with most interest in aquatic species. Regarding biotransformation biomarkers, the activity of the enzyme ethoxyresorufin *O*-deethylase (EROD) (both induction and inhibition) has been used as evidence of fish xenobiotics uptake (Hernández-Moreno *et al*., 2011), by providing indication of receptor-mediated induction of cytochrome P450-dependant monooxygenases (CYP1A subfamily) in liver phase I biotransformation (Osswald *et al*., 2013; Van der Oost *et al*. 2003). Also involved in biotransformation, glutathione *S*-transferase (GST) activity (induction) are an important family of enzymes responsible for detoxification in phase II, by catalyzing the conjugation of reduced glutathione (GSH) and electrophilic compounds (endogenous and exogenous: xenobiotics), increasing their solubility and enabling excretion (George, 1994). These enzymes are also relevant in prevention of oxidative stress (Quintaneiro *et al*., 2008). Important and commonly used biomarkers related to oxidative stress are enzymes superoxide dismutase (SOD) and catalase (CAT), related to antioxidant defense mechanisms, carrying out reduction of the toxicity of reactive oxygen species (ROS) originated from potential contaminant exposure (Fonseca *et al*., 2011b), and Lipid peroxidation levels (LPO) resulting from exposure to ROS, evidencing general cellular damage (Fonseca *et al*., 2011a). Cholinesterases (ChE) are a family of esterases, which hydrolyses carboxylic esters, divided in true cholinesterase, also known as acetylcholinesterase (AChE) and pseudo cholinesterases (PChE) or non-specific cholinesterase (Garcia *et al*., 2000; Monteiro *et al*., 2005). The AChE has an important role in the nervous system. It is responsible for the degradation of the acetylcholine neurotransmitter in cholinergic synapses, and so, if inhibited, disruption of the nerve function can

occur (Peakall, 1992). Normal function of sensory and neuromuscular systems is dependent of the nervous system (Van der Oost *et al*. 2003). Thus, effects on nerve function will affect performance of organisms and/or cause potential death (Gravato *et al*., 2010). PChE are related with prevention of AChE inhibition, reducing xenobiotic concentrations in organisms (detoxification) (Oliva *et al*., 2012). Regarding teleost fish, brain tissue contains mainly AChE (Kozlovska *et al*., 1993), while PChE is found essentially in the organism serum (Monteiro *et al*., 2005). Widely used to evaluate exposure to anticholinesterase compounds (*e.g.* organophosphates, pesticides) in several aquatic animals (Peakall, 1992), including fish, recently ChE have shown evidence of sensitivity to other contaminants such as metals (Gill *et al*., 1990; Oliva *et al*., 2012; Vieira *et al*., 2009).

1.6. Pomatoschistus microps

Pomatoschistus microps (Kröyer, 1838), also known as the common goby, is a Teleostei fish belonging to the *Gobiidae* family, which includes about 2000 species of fish (Bouchereau & Guelorget, 1997). A widespread species in estuaries, lagoons and coastal ecosystems of Europe (Vieira *et al*., 2009, 2008), its geographical distribution range from western Mediterranean regions, Morocco and southern Portugal to northern Norway coasts in the Atlantic, Wadden sea and Baltic sea (Berrebi *et al*., 2005, Leitão *et al*., 2006; Oliveira *et al*., 2012; Pockberger *et al*., 2014). It can be found in both contaminated and reference estuaries in the NW coast of Portugal (Vieira *et al*., 2008). The ability to adapt and successfully inhabit such different environments it is related to its resilience to large variations of temperature and salinity (Nyitrai *et al*., 2013) which can be a major advantage over competition with other species for resources, due to allow a greater distribution through the estuary and thus access to alternative habitat, shelter and food (Dolbeth *et al*., 2007). The *Pomatoschistus microps* live on the bottom next to the substrate along the estuaries, however several studies show higher densities in the inner regions, where water have typically brackish characteristics (Dolbeth *et al*., 2007; Leitão *et al*., 2006). This preference to brackish water may be related to higher food availability (higher organic matter levels enabling higher primary and secondary production) or to the better egg development and survival at lower salinity levels (Nyitrai *et al*., 2013). In addition

to this preference, population density and abundance also seems to follow the seasonal variations of temperature, showing migration events towards high temperature (northern areas) (Laur *et al*., 2014), and peaks in the hot seasons (spring and summer) (Dolbeth *et al*., 2007), revealing preference for higher temperatures (above 20ºC), possible due to ectothermic metabolic processes of fish (growth and reproduction) or more to egg size and development (Laur *et al*., 2014). Environmental conditions can be changed according to both anthropogenic and natural pressures that ecosystems like estuaries are usually highly subjected, with consequent alterations of the environmental parameters (*e.g.* dissolved oxygen, temperature, salinity, depth, turbidity, wind, tides circulation, currents), which can magnify natural variations, leading to changes able to influence biological processes and induce possible constrict in organisms' development and survival, mainly during egg and larvae stages, the most susceptible ones, modifying the normal life cycle of the species (Dolbeth *et al*., 2007; Nyitrai *et al*., 2013). A sedentary fish (Serafim *et al*., 2012), with short life spans, this species can live from about 1 year in Mediterranean region up to 2 years in northern Europe (Pampoulie, 2001), usually spending his entire life within an estuary (Nyitrai *et al*., 2013). According to Dolbeth *et al*. (2007) in the temperate region of Portugal, life cycle of *P. microps* is between 15 to 17 months. An iteroparous batch spawner species (ability to produce and release more than one batch of eggs per reproductive season (Laur *et al*., 2014)), has three recruitment cohorts known per year: January/February, April and June/July (Dolbeth *et al*., 2007; Leitão *et al*., 2006; Nyitrai *et al*., 2013). Regarding the parental care, males and females have distinct roles. While females only have the function of lay their demersal eggs, males choose the nest location, exhibiting territorial behavior and becoming very competitive when suitable locations are limited and build it so one, or more females, lay their eggs and males proceed their main responsibilities of taking care and protecting the eggs until they develop and hatch to larval form (Bouchereau & Guelorget, 1997; Leitão *et al*., 2006), generally with 2.5 mm long, and its pelagic independent life begins. Between one/two months later, organisms undergo a last transformation and adopt a benthic habit, starting to live on the bottom (Laur *et al*., 2014; Leitão *et al*., 2006), reaching 64 mm long maximum as adults (Pockberger *et al*., 2014). Populations of *P. microps* are in general young, with a small part represented by older individuals (1 year old fish) (Dolbeth *et al*., 2007; Leitão *et al*., 2006). The temperature can be considered a very important regulatory factor, with the ability

to influence the reproductive strategy and their success, the number and duration of recruitments (Dolbeth *et al*., 2007). For example, shorter reproductive seasons in populations of Northern Atlantic in relation to populations in Mediterranean region (Leitão *et al*., 2006), and earlier sexual maturity in Mauguio Lagoon-Mediterranean region (Bouchereau & Guelorget, 1997). Spawning occurs preferentially at temperatures between 15ºC to 20ºC and high egg survival is above 20ºC (Leitão *et al*., 2006). The great adaptability of *P. microps* already mentioned before can be once again an advantage in the reproductive effort, allowing the adaptability towards different ecosystems or unpredictable environment variations, managing energy used in the process, longer reproduction seasons, several spawning periods, to maximize survival chances (Dolbeth *et al*., 2007; Pampoulie, 2001). In relation to its diet, the common goby is an omnivorous fish species, exhibiting a broad spectrum of prey items (Dolbeth *et al*., 2007). Feeding on about 60 different prey species, their diet as goby larvae consists of zooplankton, when became juveniles they start to feed on meiofauna, *e.g.* hapacticoid copepods, ostracods, and change gradually to macrofauna, bigger preys, feeding of items of mollusca, crustacea (amphipoda, isopoda), polychaeta, bivalvia, insecta and small teleost, as become larger and reaches the adult stage (Jackson & Rundle, 2008; Leitão *et al*., 2006; Pockberger *et al*., 2014). Several studies determine polychaetes, mysids, isopods and bivalve siphons as the preferred prey. This diet alteration overlap with change of individuals energy needs and occurs independently the availability of prey, with observed reduced fitness when this shift fail or optimal prey is not available (Jacskson & Rundle, 2008). Apart being predators, common gobies are relevant prey for bigger predators to (Pockberger *et al*., 2014).

According to some authors, the *P. microps* has a really important role in coastal and estuarine trophic webs as make the energy flow between and inside this ecosystems, as intermediate predator, making the connection between macro-, meio- and micro-benthos (consumer) and larger fish species and several birds (prey) (Dolbeth *et al*., 2007; Monteiro *et al*., 2005; Oliveira *et al*., 2012; Pockberger *et al*., 2014; Quintaneiro *et al*., 2008; Serafim *et al*., 2012; Vieira *et al*., 2008, 2009). Due to characteristics such as sensitivity to ecological variations (*e.g*. temperature, salinity) (Berrebi *et al*., 2005), wide geographical distribution, high abundance and fecundity, easy maintenance and handling (Quintaneiro *et al*., 2008) and most important, key function as intermediate predator, has been

considered as an adequate bioindicator in monitoring studies and suitable test organism (Quintaneiro *et al*., 2008; Vieira *et al*, 2008), already successfully used in several laboratory assays and field studies (Monteiro *et al*., 2005, 2006, 2007; Oliveira *et al*., 2012, 2013; Vieira *et al*., 2008, 2009). The possibility to evaluate/assess the ecosystems conditions without capture the full complexity of the system and cause possible damage is the great advantage of use fish as bioindicators of environmental changes (Nyitrai *et al*., 2013).

1.7. Objectives and outline of the thesis

The main objective of this Thesis was to investigate the toxic effets of nickel and microplastics in early juveniles (0^+ group) of *Pomatoschistus microps* from wild populations of Minho and Lima river estuaries, after exposure to the agents individually and in combination, regarding previous distinct long-term exposure to different levels of environmental contamination in early development stages. Especially, it was intended to test the following hypotheses: (i) Juveniles from estuaries with different levels of environmental contamination have different sensibilities to nickel; (ii) the presence of microplastics in water influences the toxicity of nickel towards juveniles. This particular specie were selected due to be consider a good test organism (*e.g.* Monteiro *et al*., 2005, 2006; Oliveira *et al*., 2012, 2013; Vieira *et al*., 2008, 2009).

The Thesis includes four main chapters: Chapter 1 – Introduction; Chapter 2 – Material and Methods; Chapter 3 – Results and Discussion and Chapter 4 – Conclusions and Future Perspectives. In Chapter 1 an introduction to the problem of environmental pollution, some useful concepts to a better understanding about the theme and the objective and outline of the Thesis is made. In Chapter 2 it is described the experimental procedures performed so that the purpose of this Thesis could be achieved. Chapter 3 corresponds to the description, interpretation and validation of the obtained results. Finally, in Chapter 4 are the conclusions and future perspectives, where the main findings of the work done are highlighted.

2. Material and Methods

2.1. Test substances and other chemicals

Nickel chloride hexahydrate ($NiCl_2.6H_2O$) (CAS number: 7791-20-0; Puriss p.a. ≥98%) and fluorescent polyethylene red microspheres (1-5µm), hereafter indicated as MP, used as test substances were purchased from *Sigma-Aldrich* (Germany) and *Cospheric innovations in Microtechnology* (USA), respectively. Biorad reagent used for protein determinations was acquired from *Bio-Rad* (Germany). All the other chemicals used for biomarkers analysis were obtained from *Sigma-Aldrich* (Germany) or *Merck* (Germany). The artificial salt water (ASW) used on fish maintenance in laboratory and test medium in bioassays was prepared by dissolving marine salt *Ocean fish,* purchased from *Prodac international S.r.l.* (Italy), in deionized (DI) water and adjusting the salinity to 18 g/l.

2.2. Fish sampling, transport and maintenance in laboratory

P. microps juveniles with a total length of less than 2.5 cm were captured at low tide using a hand operated net, in two different estuaries: the Minho river estuary and the Lima river estuary, both in NW of Portugal. The geographic coordinates of the sampling sites are: 41º53'31"N, 8º49'28"W (Minho river estuary) and 41º41'11.41"N, 8º49'20.42"W (Lima river estuary). The Minho river estuary has a total area of about 3.4 km^2 (Maretec, n.d.), mean depth of 2.6 m (Costa-Dias *et al.*, 2010b) and is classified as a mesotidal with semidiurnal tide estuary (Quintaneiro *et al.*, 2008). It has high ecological value and therefore is included in the Nature 2000 Network (Pinto & Martins, 2013). Characterized by low environmental pollution (Ferreira *et al.*, 2003), it has been considered and used as a reference area in several previous studies performed with this particular species (*e.g.* Oliveira *et al.*, 2012; Monteiro *et al.*, 2005, 2006, 2007; Vieira *et al.*, 2008, 2009). The Lima river estuary is located near a city with about 46 000 inhabitants (Viana do Castelo), with a total area of about 6 km^2 (Costa-Dias *et al.*, 2010a) and a dredged channel of 10 m, also classified as a semidiurnal tide mesotidal estuary (Ramos *et al.*, 2010). Considered as an urban-industrialized estuary by Almeida *et al.* (2011), it is exposed to several anthropogenic pressures

such as a cellulose factory in upper estuary, input of agricultural runoff and urban and industrial sewage, navigation activities associated to the large shipyard, marina, and important commercial/fishing harbour in lower estuary and constant dredging of the navigational channel, with possible remobilization of contaminants (Almeida *et al.*, 2011; Azevedo *et al.*, 2013). According to the Water Framework Directive (WFD), in 2010, the Lima river estuary had a moderate ecological status (Costa-Dias *et al.*, 2010a). These two estuaries were chosen due to the different levels and sources of contamination, which could affect/modify the performance of the individuals, enabling a comparison.

After being collected, *P. microps* specimens were transported to the laboratory within the lowest time interval possible in thermally isolated 30 L containers with water from the sampling site at environmental temperature and air supply. In the laboratory, fish were submitted to an acclimation period of two weeks, before the start of the toxicity test, in 80 L aquaria with ASW prepared as indicated in section 2.1. A digital seawater refractometer HI 96822, *Hanna Instruments* (USA) was used to measure the water salinity. External filters pumps (EHEIM filters, *EHEIM GmbH & Co. KG*, Germany) were used to maintain the water clean and air supply was provided (**Figure 1**). About two hundred fish were maintained in each aquarium. The aquaria were in a room with a photoperiod of 14h light: 10h dark and room temperature between 17ºC to 22ºC. In order to reduce fish stress as much as possible, the medium was gradually changed three times a week and fish were fed *ad libidum* in a daily basis with commercial fish food *Tropic Mix* (*Aquapex Produtos*, Portugal).

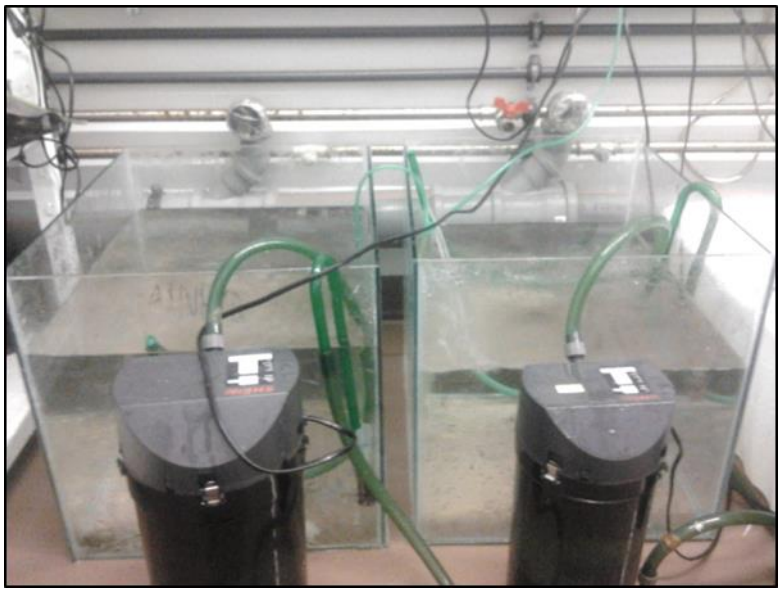

Figure 1 – Aquaria used to maintain the juveniles of *P. microps* in the laboratory during the acclimation period.

2.3. Investigation of the possibility of using a spectrophotometric method to determine the concentrations of nickel in the bioassays

It is convenient to check the actual concentrations of Ni and its potential decay during the bioassays. Therefore, the possibility of using the spectrophotometer method of DerVartanian & Chenoweth (2000) in our experimental conditions was investigated.

According to this technique, suitable to both protein and non-protein solutions, the dithiothreitol (DTT) and phosphate, when in combination, will form ligands which in turn will react with Ni, forming Ni phosphothiocomplexes spectrophotometrically detectable at 465 nm (DerVartanian & Chenoweth, 2000). So, two initial solutions with a nominal concentration of Ni equal to 100 mg/l were prepared, one in ASW (section 2.1.), the test medium of the bioassays, and another one in ultra-pure (u.p.) water. Following the DerVartanian & Chenoweth (2000) technique, 1 ml of each Ni solution was transferred to individual test tubes, where 0.1 ml of sodium phosphate buffer (0.1 M, pH = 7.5) and 0.1 ml of DTT (0.01 M) was added. Test tubes were immediately inverted to mix reagents and let it stand for ten minutes in order to allow the formation of the Ni phosphothiocomplexes and their characteristic red-brownish colour to appear. One ml of each solution was put in a quartz cuvette and two full absorption (200 to 900 nm) spectra were made (Spectramax M2^{e}, *Molecular devices, LLC,* USA). Then, a calibration curve in ASW was prepared by serial dilution of the initial solution of Ni 1:2 (v/v), with the same procedure described above performed in each one. For comparative purposes, a similar curve was made in u.p. water from the initial Ni solution in u.p. water. The nominal concentrations of Ni of all the solutions prepared were: 3.1, 6.3, 12.5, 25.0, 50.0 and 100 mg/l (**Figure 2**). The absorbance of these solutions was measured at 470 nm in the same spectrophotometer. The solutions were not kept for further Ni decay evaluation due to the precipitation of the compound after fifteen minutes (DerVartanian & Chenoweth, 2000). The degree of correlation of the two variables (logarithm of the nominal Ni concentrations and corresponding absorbance at 470 nm) was investigated using the Pearson correlation coefficient (r). Then, the absorbance values were plotted against the corresponding logarithm of the nominal concentrations of Ni and a linear model was fitted to the data, using absorbance values as independent variable and Ni logarithm concentrations as dependent variable.

The equation of the model was used to determine the actual concentrations of Ni in test media from the corresponding absorbance readings. The potential decay of Ni in test media was investigated from absorbance readings at 0, 24, 48, 72 and 96h.

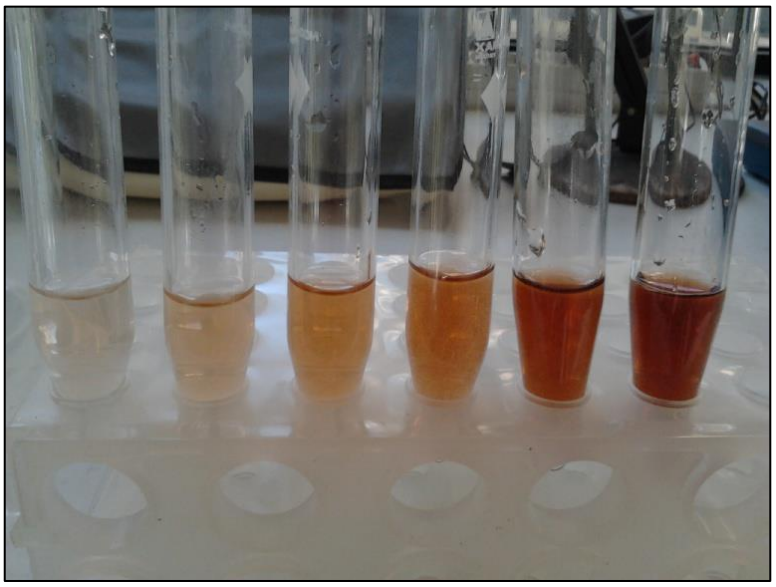

Figure 2 – Nickel phosphothiocomplexes formation with color appearance in nickel spectrophotometrically analyzed samples. Nominal concentrations of nickel (reader left to right): 3.1; 6.3, 12.5, 25.0, 50.0, 100 mg/l.

2.4. Pre-screening of nickel toxicity to *P. microps* juveniles

In a first phase of the study, a first-screening toxicity assay was carried out to optimize the procedures and techniques, and to determine the range of Ni toxicity to the tested species. The bioassay followed the general OECD guidelines for fish acute bioassays (OECD, 1992) with some differences as explained bellow. The fish used in this bioassay were collected in the Spring.

All the glass material used was carefully washed with acid (HNO_3 10%) and distilled water before the bioassay. A stock solution of Ni chloride hexahydrate in u.p. water was prepared with a nominal Ni concentration of 100000 mg/l. From this stock solution, a first test solution with a concentration of 50 mg/l of Ni was prepared by diluting 1 ml of the stock solution into ASW prepared as indicated in section 2.1. (final volume of 2000 ml). This solution was then serial diluted 1:2 (v/v) in ASW to obtain 5 additional test concentrations. The process was repeated

until obtaining the volume of each solution needed for the bioassay. Thus, the tested concentrations of Ni were: 1.6, 3.1, 6.3, 12.5, 25.0, and 50.0 mg/l of Ni. These concentrations were chosen based in a review of the literature on fish bioassays with Ni. A control treatment (ASW only) was included in the experimental design.

After the two weeks acclimation period, twenty one fish were randomly selected for use in this bioassay. Because this was a preliminary test, following the animal experimentation "3R's" principle (Reduction, Replacement and Refinement), only 3 fish from the Minho river estuary were used per treatment. Also, to reduce the stress of handling, fish were only weighted and measured at the end of the exposure period (after the predatory performance assay and before biomarkers determinations). Fish were weighted in an analytical balance (Kern ABS-N, *KERN & SOHN GmbH,* Germany) and measured with a digital caliper (*Electronic Digital Caliper*, China). Fish were exposed individually for 96h in glass beakers of 1 L with 500 ml of test medium, with continuous air supply and sealed to avoid potential losses of the test substance or the increase of its concentration due to water evaporation, and to prevent contamination (**Figure 3**). No food was provided during the bioassay and feeding was stopped 24h before the beginning of the experiment. The photoperiod was maintained the same of acclimation period (14h light: 10h dark) and test medium was not changed. At the beginning of the bioassay and at each 24h, the following parameters were determined in test media: temperature, pH and dissolved oxygen using a HACH 40d probe (*Hach Company*, CA, USA) and salinity using a digital seawater refractometer HI 96822 (*Hanna Instruments*, USA). Fish were observed as much as possible and dead fish were removed as soon as noticed. Mortality, one of the effect criteria, was recorded at each 24h intervals. Fish were considered dead when no visible body and opercular movement were observed and were immediately removed. The other effect criteria was post-exposure predatory performance of fish (hereafter indicated as predatory performance), assessed immediately after the 96h of exposure (see section 2.7.). After the predatory performance trials, fish were put back in their original test media, where they recovered for 2h before being euthanized by decapitation for further biomarkers determination, as explained in section 2.9. The biomarkers analysed in this bioassay were acetylcholinesterase (AChE) activity and lipid peroxidation (LPO) levels (procedures indicated in section 2.9.).

Figure 3 – Fish exposure to different concentrations of nickel. Green tubes are part of the air supply system.

2.5. Comparative effects of nickel alone in juvenile fish from the Minho and Lima estuaries

The objectives of this bioassay were to investigate the effects of Ni on *P. microps* juveniles, and to compare the sensitivity of fish from the populations of Minho and Lima estuaries to this common environmental contaminant. This is important because the environmental conditions during previous developmental phases, including long-term exposure to different levels of environmental contamination, may influence the sensitivity of juveniles to Ni. In general, the bioassay was carried out as indicated in section 2.4. with some modifications that are indicated below. The fish used in this bioassay were collected in the Spring.

A stock solution of Ni chloride hexahydrate (nominal concentration of Ni = 200000 mg/l) in u.p. water was prepared. From this solution, the first tested concentration was prepared by dilution of one ml in ASW (Ni nominal concentration = 100 mg/l) (final volume = 2000 ml). This solution was serial diluted (1.2, v/v) in ASW to obtain the other test solutions: 50.0, 25.0, 12.5, 6.3 and 3.1 mg/l of Ni. The tested concentrations of Ni were increased relatively to the first screening assay since no mortality was recorded during the 96h experiment. Fish from the control group were exposed to ASW only. Eighteen

fish (nine from each estuary), randomly selected among those in acclimation, were used per treatment. The fish were individually exposed as previously indicated (section 2.4.). All the other conditions and procedures, including water parameters measurements were as previously indicated. Effect criteria were: mortality, predatory performance, lipid peroxidation (LPO) levels, and the activity of the enzymes acetylcholinesterase (AChE), glutathione *S*-transferase (GST) and ethoxyresorufin *O*-deethylase (EROD). The parameters and mortality were determined as indicated in section 2.4. and 2.7. and 2.9. for predatory performance and biomarkers, respectively. The actual concentrations of Ni in test media were determined from the absorbance readings (at 470 nm) at the beginning of the bioassay (time 0h) using the equation of the linear model (section 2.3.). The potential decay of Ni during the bioassay was investigated from the absorbance readings of tested media samples collected at time 0, 24, 48, 72 and 96h. In this bioassay room temperature and humidity were also measured with a DATA LOGGER (PCE-HT 71 Humidity/Temperature, *PCE instruments UK Ltd*, UK) device. Values were recorded between 19.9 ºC and 23.2 ºC for temperature and between 34.0 Pa and 46.4 Pa for humidity.

2.6. Influence of microplastics on the toxicity of nickel to juvenile fish from the Minho and Lima estuaries

The objective of this third bioassay was to assess the influence of MP on the toxicity of Ni. The fish used in this bioassay were collected in the Summer.

The bioassay was carried out as indicated in sections 2.4. and 2.5. with some differences that will be further indicated. The following treatments were tested: control (ASW only), MP alone (0.184 mg/l), and six mixture treatments, namely, 100 mg/l of Ni + 0.184 mg/l of MP, 50.0 mg/l of Ni + 0.184 mg/l of MP, 25.0 mg/l of Ni + 0.184 mg/l of MP, 12.5 mg/l of Ni + 0.184 mg/l of MP, 6.3 mg/l of Ni + 0.184 mg/l of MP, and 3.1 mg/l of Ni + 0.184 mg/l of MP. The tested concentrations of Ni were prepared as previously described (section 2.5.). A stock solution of MP in u.p. water was prepared (92 mg/l). The appropriate amount (*e.g.* 1ml in 500ml) of this solution was pipetted directly to the test media to obtain the concentration of MP tested alone and in mixture with Ni. Eighteen fish (nine from each estuary), randomly selected among those in acclimation, were used per

treatment. Additionally, test beakers similar to those used for the MP alone and Ni treatments but without fish (3 replicates each) were included to investigate the potential MP and Ni decay in test media in the absence of fish. All the other conditions, procedures, and effect criteria were similar to those previously described (sections 2.4. and 2.5.). In addition, test media samples were collected at time 0, 24, 48, 72 and 96h to assess the potential decay of MP during the bioassay. The fluorescence of each sample was measured in a spectrofluorometer (Jasco FP-6220, *Jasco Analytical Instruments*, USA), with a wavelength excitation and emission of 470 nm and 588 nm, respectively. Room temperature and humidity were measured as indicated in section 2.5. Values were recorded between 19.9 ºC and 23.2 ºC for temperature and between 34.0 Pa and 46.4 Pa for humidity.

2.7 Post-exposure predatory performance assay

The predatory performance of *P. microps* juveniles was assessed immediately after the end of the exposure period (96h), for each fish individually. A procedure previously developed and validated in the scope of the project SIGNAL was used. Briefly, each fish was transferred from its original test beaker to a prey-exposure chamber (**Figure 4**) containing 300 ml of ASW (section 2.1.) where it was left for five minutes. After these five minutes, thirty nauplii of *Artemia franciscana* (< 24h old) were added to the prey-exposure chamber and the number of nauplii ingested by the fish was recorded for three minutes. Then, the fish was removed from the prey-exposure chamber (back to its original test beaker) and the number of nauplii remaining in the chamber was determined. The *Artemia franciscana* was previously counted with aid of a Nikon stereomicroscope (Nikon SMZ800, *Nikon Instruments Inc.*, USA). The number of the prey ingested by the fish (IP) was determined by subtracting the number of nauplii remaining in the prey-exposure chamber (B) from the number of prey offered to the fish (A = 30). The predatory performance of each fish was expressed as the percentage of the prey ingested by fish relatively to the initial number of prey offered: IP (%) = (A-B) * 100 / A. The ASW in the prey-exposure chamber was changed whenever a new fish was tested. After this predatory performance assay, fish were put back in their original test solutions, where they stood for 2h before further euthanasia for further biomarkers determination, as explained in section 2.9.

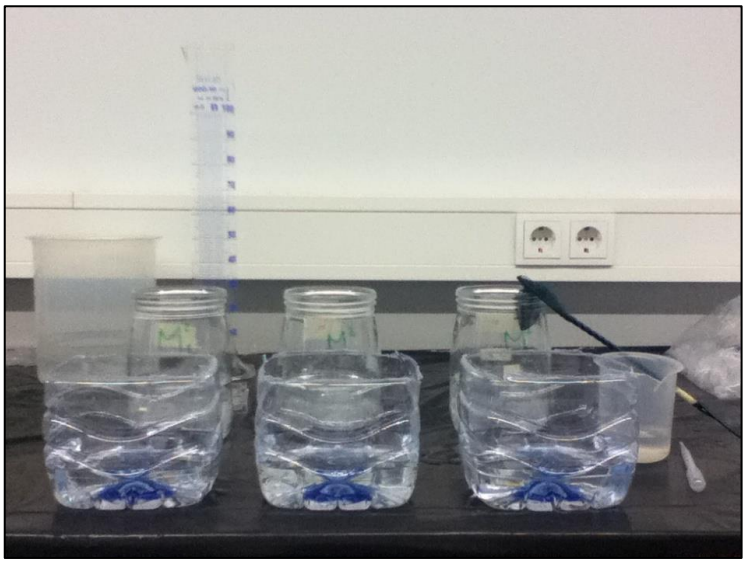

Figure 4 – Prey-exposure chambers used in the post-exposure predatory performance assay.

2.8. Morphometric parameters and preparation of biological material for biomarkers

After 2h of resting in the original test beakers, each fish was weighted and measured, as indicated in section 2.4. (total length: from 1.8 to 2.5 cm). Then, fish were euthanized by decapitation under ice-cold induced anaesthesia. No other anaesthetics were used to avoid potential interaction with biomarkers, especially AChE. From each fish, the head and the body were isolated separately on ice. Then, the head of each fish was put into 1 ml of cold phosphate buffer (0.1M, pH = 7.2), while the body was put into 2 ml of cold phosphate buffer (0.1M, pH = 7.4). The samples were then homogenised on ice for between fifteen and twenty seconds, using an Ystral D-79282 homogenizer (*Power Technologies Inc*, Germany). In some exceptional cases, when the specimens were slightly smaller, half the normal amount of buffer was used. Head homogenates were centrifuged (refrigerated centrifuge Eppendorf 5810R, *Eppendorf AG, Germany*) for three minutes at 3300 x g and 4ºC. The supernatants were carefully collected and preserved at -80ºC (SANYO Ultra Low Temperature VIP Plus Freezer*, Electric Co., Ltd*, Japan) until further AChE activity analysis. From each body homogenate, 0.250 ml were put into a microtube containing 0.004 ml of butylated

hydroxytoluene (BHT) in methanol (4 %) (solution previously performed by dissolving 0.4 g of BHT in 10 ml of methanol) and stored at -80ºC for further determination of LPO levels. The remaining body homogenates were centrifuged for 20 minutes at 10000 x g and 4ºC. Each supernatant was carefully recovered, divided in 0.2 ml samples and stored at -80ºC for further GST and EROD activity determinations.

2.9. Analysis of biomarkers

The AChE activity was determined in samples obtained from individual heads as previously indicated (section 2.8.). GST activity and LPO levels were determined in samples obtained from individual fish (section 2.8.). For EROD activity analysis, pools of three samples from three different fish (exposed to the same treatment) were used because the activity detected in individual samples was very low (preliminary experiences done out of the scope of this thesis).

First, protein content of all the samples for biomarkers was determined by the Bradford technique (1976) adapted to microplate (Frasco & Guilhermino, 2002) using ɣ-bovine's globulins (*Sigma-Aldrich*) as standard protein. In short, samples were diluted at a ratio of 20 µl of sample to 180 µl of phosphate buffer (10 x dilutions). Phosphate buffer with 0.1M, 7.2 pH for head samples and 0.1M, 7.4 pH for body samples. Then, 10 µl of each diluted sample were transferred for each three correspondent wells (triplicate samples) and added 250 µl of Bio-Rad solution. The microplates were placed in a mixer during fifteen minutes and absorbance measured in a microplate reader (BIO-TEK, POWERWAVE 340, *Biotek*, USA) at 600 nm and 25ºC. Based in previous studies performed by the team with this particular species, protein content of samples for AChE and GST activity determination were normalized to 0.5 mg/ml before the analysis.

AChE activity was determined by the Ellman's method (Ellman's *et al*., 1961), adapted to microplate (Guilhermino *et al*., 1996). Thus, 50 µl of sample was transferred for the microplate correspondent three wells of each sample and added 250 µl of the reaction solution previously performed (0.2 ml of acetylthiocholine and 1 ml of DTNB (5-5'-dithio-bis-(2-nitrobenzoic acid)) solution in 30 ml of phosphate buffer (0.1M, pH = 7.2)). Degradation rate of AChE was

assessed at 412 nm and 25ºC, during five minutes through assessing the increase of the yellow color resultant of the combination between the thiocholine and the DTNB. At the end, protein content was confirmed and enzymatic activity were calculated with these final protein values and expressed as nmol of substrate hydrolysed per minute per mg of protein. According to Monteiro *et al*. (2005) the head homogenate soluble fraction of the *P. microps* contains mainly AChE.

As for GST activity determination, the approach of Habig *et al*. (1974) was followed, adapted to microplate (Frasco & Guilhermino, 2002). Thus, 50 µl of sample was transferred for the microplate correspondent wells and added 250 µl of the reaction solution of CDNB (1-chloro-2,4-dinitrobenzene) previously performed (1.5 ml of CDNB and 9 ml of GSH in 49.5 ml of phosphate buffer, 0.1M, pH = 6.5). The absorbance of the reaction (GSH and CDNB combination) was recorded at 340 nm and 25ºC during five minutes. At the end, protein content was confirmed and enzymatic activity were calculated with these final protein values and expressed as nmol of substrate hydrolysed per minute per mg of protein.

Regarding the EROD, activity was quantified using the fluorimetric method described by Burke & Mayer (1974) with some modifications. First, 100 µl of three samples (of the same treatment) were put in a quartz cuvette and added 1000 µl of reaction buffer previously performed (1.5 ml of ethoxyresorufin in 49.5 ml of phosphate buffer (0.1M, pH = 7.4)) and 10 µl of NADPH solution (0.0166 g NADPH in 2 ml of phosphate buffer (0.1M, 7.4 pH). The production of resorufin was measured with a spectrofluorometer, Jasco FP-6220 (*Jasco Analytical Instruments*, USA), immediately after NADPH were added, at an absorbance kinetic reading of 530 nm and 585 nm (25ºC) (excitation and emission wavelengths, respectively), during two cycles of thirty seconds. Enzymatic activity were calculated with the protein content values initially determined and expressed as nmol of substrate hydrolysed per minute per mg of protein.

The assessment of the lipid peroxidation (LPO) levels was performed according to Ohkawa *et al*. (1979) by the quantification of the thiobarbituric acid reactive substances (TBARS) in body tissue homogenates. Thus, 200 µl of the homogenates were transferred to 15 ml tubes and were added 1000 µl of trichloroacetic acid (TCA) solution, 800 µl of tris(hydroxymetil)-aminomethane

(Tris-HCL) solution and 1000 µl of thiobarbituric acid (TBA) solution to each sample. Two controls were performed with 200 µl of phosphate buffer (0.1M, pH = 7.4) in the same conditions. The tubes were then incubated in a water bath during sixty minutes at 100ºC. Two ml of each final sample were transferred to microtubes and centrifuged at 11500 rpm and 25ºC, during five minutes. To finalize, absorbance were measured with a spectrophotometer, JENWAY model 604 UV/VIS (*Bibby Scientific Limited*, UK), at 535 nm. LPO levels were calculated with the protein content values initially determined and expressed as nmol of TBARS formed per mg of protein.

2.10 Statistical analysis

First, data was tested for normality using the Kolmogorov-Smirnov normality test and homogeneity of variance with the Bartlett's test. Data transformations were performed when required, logarithmic transformation of nominal concentrations of Ni (Ni concentrations standard curve) and arcsine transformation of the square root of the percentage values of the predatory performance (Zar, 1999). A linear regression model was used to estimate the actual concentrations of Ni in test media and Pearson correlation coefficient used to measure the correlation between variables. Median lethal concentration (LC_{50}) and half maximal effective concentration (EC_{50}), and respective 95% confidence limits (95% CL), were calculated by probit analysis. To make a simple comparison between different treatments and the control group in the predatory performance and biomarkers of the pre-screening bioassay, were used a one-way analysis of variance (ANOVA) followed by the Dunnett's test. In order to compare biomarkers activities and the predatory performances among estuaries (origin) and different exposure treatments, were used a two-way analysis of variance (ANOVA) with interaction between the two factors (Origin and Treatments) followed by the Tukey's multiple comparison test. When significant differences were identified among treatments, a one-way analysis of variance (ANOVA) and Tukey's post hoc tests were applied.

All statistical analyses were performed using the SPSS 20 software package and differences were considered statistically different when $p \leq 0.05$.

3. Results and Discussion

3.1. Pre-screening bioassay

The results of temperature, pH, dissolved oxygen and salinity measured in test media during the 96h pre-screening bioassay are shown in **Table 1**. Salinity values remained stable in the 18 g/l and pH changed between 8.4 and 8.6 units. The temperature variation was less than 2ºC. Dissolved oxygen was always above the 60% as required by OECD to the bioassay to be valid (OECD, 1992). The total length and weight (mean ± standard deviation (SD)) of the fish were 1.7 ± 0.1 cm and 0.052 ± 0.013 g, respectively. No mortality was recorded in any of the treatments. Significant differences in the predatory performance of fish exposed to different treatments were found ($F_{(6,14)}$ = 6.114; $p \leq 0.05$), with a significant reduction of prey ingestion observed in fish exposed to 25.0 and 50.0 mg/l of Ni relatively to the control group (**Figure 5**). No significant differences in AChE activity ($F_{(6,14)}$ = 0.747; $p > 0.05$) (**Figure 6A**) nor in lipid peroxidation (LPO) levels ($F_{(6,14)}$ = 0.622; $p > 0.05$) (**Figure 6B**) among treatments were found, indicating that in the range of concentrations tested, Ni does not induce anticholinesterase nor lipid peroxidation damage, respectively. Regarding the LPO levels, abnormal low values are indicated in treatment with 6.3 and 25.0 mg/l of Ni, perhaps due to a lack of experience in performing the technique.

Therefore, in order to assess the potential effects of Ni and to determine the LC_{50} in this species, the treatments concentrations were increased in the following bioassays.

Table 1 – Abiotic parameters measured in test media of the 96h pre-screening bioassay carried out to investigate the effects of nickel on *Pomatoschistus microps* from the Minho river estuary. The determinations were made at the beginning (0h) and at each 24h. Values are the mean with the corresponding standard deviation (SD). DO – water dissolved oxygen. Ni – concentrations of nickel.

Treatment (Ni mg/l)	**Exposure** (hours)	**Temperature** (ºC)	**Salinity** (g/l)	**pH**	**DO** (mg/l)
0	0h	16.20 ± 0	18 ± 1	8.53 ± 0.01	10.60 ± 0.27
	24h	16.27 ± 0.12		8.40 ± 0.12	10.17 ± 0.09
	48h	15.60 ± 0.26		8.46 ± 0.06	10.29 ± 0.06
	72h	16.17 ± 0.59		8.60 ± 0.03	10.09 ± 0.03
	96h	16.17 ± 0.23		8.56 ± 0.02	9.95 ± 0.13
	|96h-0h|	-0.03 ± 0.23	0	0.03 ± 0.01	-0.65 ± 0.14
1.6	0h	16.53 ± 0.15	18 ± 1	8.53 ± 0.01	9.90 ± 0.14
	24h	16.53 ± 0.35		8.54 ± 0.02	10.01 ± 0.09
	48h	16.03 ± 0.35		8.53 ± 0.01	9.70 ± 1.29
	72h	16.53 ± 0.35		8.55 ± 0	9.99 ± 0.07
	96h	16.30 ± 0.10		8.54 ± 0.01	9.92 ± 0.10
	|96h-0h|	-0.23 ± 0.05	0	0.01 ± 0	0.02 ± 0.04
3.1	0h	16.67 ± 0.06	18 ± 1	8.53 ± 0.01	9.94 ± 0.02
	24h	16.40 ± 0.26		8.54 ± 0.01	9.95 ± 0.08
	48h	15.87 ± 0.45		8.54 ± 0.01	10.01 ± 0.32
	72h	16.43 ± 0.25		8.55 ± 0.01	10.01 ± 0.06
	96h	16.47 ± 0.25		8.50 ± 0.02	9.81 ± 0.06
	|96h-0h|	-0.20 ± 0.19	0	-0.03 ± 0.01	-0.13 ± 0.04
6.3	0h	16.37 ± 0.31	18 ± 1	8.53 ± 0.01	10.01 ± 0.08
	24h	16.07 ± 0.45		8.52 ± 0.02	9.93 ± 0.31
	48h	15.70 ± 0.40		8.52 ± 0.01	10.07 ± 0.35
	72h	16.30 ± 0.20		8.55 ± 0	10.04 ± 0.06
	96h	16.60 ± 0.10		8.52 ± 0.01	9.90 ± 0.15
	|96h-0h|	0.23 ± 0.21	0	-0.01 ± 0	-0.11 ± 0.07
12.5	0h	16.50 ± 0.10	18 ± 1	8.51 ± 0.01	9.95 ± 0.08
	24h	16.13 ± 0.15		8.53 ± 0.01	10.00 ± 0.09
	48h	15.90 ± 0.20		8.54 ± 0	10.10 ± 0.07
	72h	16.43 ± 0.15		8.56 ± 0.01	10.02 ± 0.05
	96h	16.63 ± 0.12		8.52 ± 0.01	9.81 ± 0.06
	|96h-0h|	0.13 ± 0.02	0	0.01 ± 0	-0.14 ± 0.02
25.0	0h	16.30 ± 0.30	18 ± 1	8.51 ± 0.02	10.08 ± 0.04
	24h	15.50 ± 0.44		8.55 ± 0.01	10.20 ± 0.04
	48h	15.37 ± 0.32		8.53 ± 0.01	10.22 ± 0.18
	72h	15.90 ± 0.30		8.55 ± 0.02	10.12 ± 0.12
	96h	16.37 ± 0.21		8.53 ± 0.02	10.01 ± 0.05
	|96h-0h|	0.07 ± 0.09	0	0.02 ± 0	0.07 ± 0.01
50.0	0h	16.20 ± 0.10	18 ± 1	8.51 ± 0.01	10.08 ± 0.03
	24h	15.83 ± 0.25		8.52 ± 0.02	10.04 ± 0.18
	48h	15.57 ± 0.21		8.53 ± 0.01	10.23 ± 0.03
	72h	16.17 ± 0.21		8.53 ± 0.01	9.99 ± 0.05
	96h	16.87 ± 0.12		8.52 ± 0.01	9.88 ± 0.19
	|96h-0h|	0.67 ± 0.02	0	0.01 ± 0	-0.20 ± 0.16

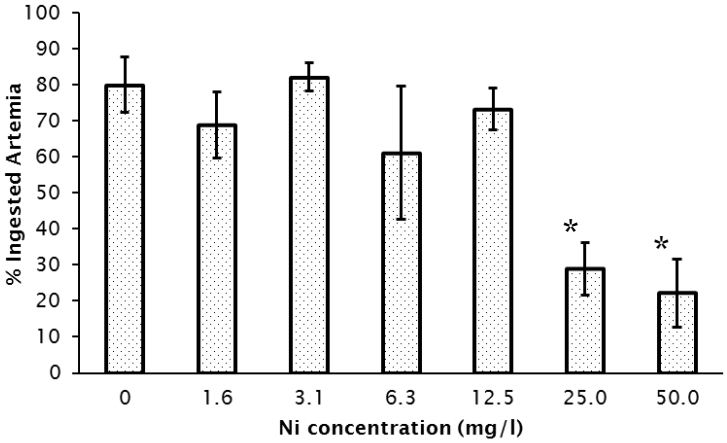

Figure 5 – Post-exposure predatory performance of *Pomatoschistus microps* juveniles from the Minho river estuary after 96h of exposure to artificial salt water only (0 - control group) or to different nickel (Ni) concentrations. The predatory performance is expressed as the percentage of prey (*Artemia franciscana* nauplii) ingested by fish relatively to the control group (0). The results are expressed as the mean of three fish per treatment with the corresponding standard error bars. * - Significantly different from the control group as indicated by one-way analysis of variance and the Dunnett's test ($p \leq 0.05$).

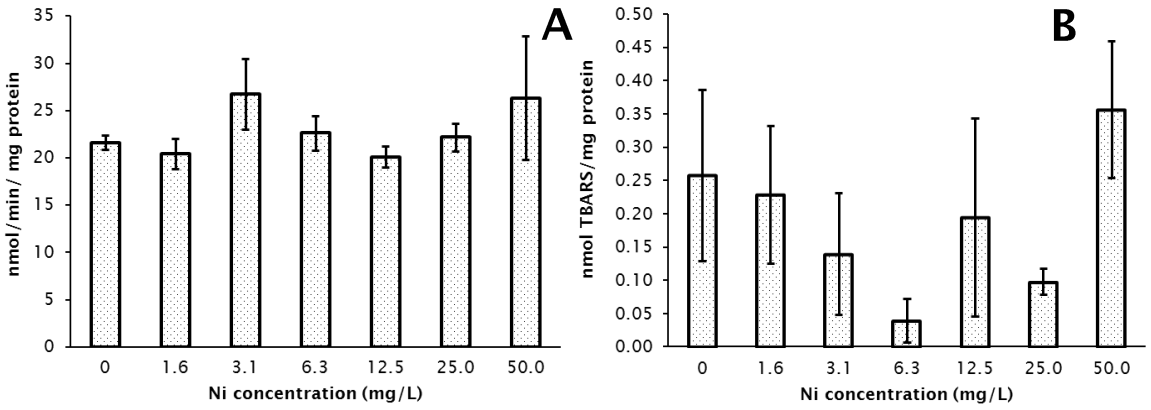

Figure 6 – Acetylcholinesterase (AChE) activity (**A**) and lipid peroxidation (LPO) levels (**B**) of *Pomatoschistus microps* juveniles from the Minho river estuary after 96h of exposure to artificial salt water (0 – control group) only or to different concentration of nickel (Ni). The values are the mean of three fish per treatment with corresponding standard error bars.

3.2. Comparative effects of nickel alone in juveniles fish from the Minho and Lima estuaries

3.2.1. Measurement of nickel concentrations and its potential decay in the bioassay

Figure 7A and **B** represent the obtain scans in u.p. water and ASW, respectively, of Ni and allow to identify a UV absorbance peak at 470 nm in both. Slightly higher than the theoretical (465 nm), according to the literature, this could happen due to a larger amount of metal ions present in the samples relatively to DTT concentrations. This shift does not interfere in Ni determination (DerVartanian & Chenoweth, 2000).

In the range of concentrations tested, the absorbance at 470 nm shows a significant correlation with the Ni logarithm concentration (Pearson coefficient correlation, r= 983, $p<0.05$, N=18 in u.p. water and r=974, $p<0.05$, N=18 in ASW) (**Figure 8A** and **B**). Therefore, the regression equation obtained by fitting a linear regression model to the data could be used to estimate the actual Ni concentrations in the test media from their absorbance at 470 nm. **Table 2** indicates the actual Ni concentrations determined in test media, calculated from the linear regression equation shown in **Figure 8B.** Since the difference between nominal and actual concentration was always less than 20%, according to OECD (1992) nominal concentrations can be used to indicate the tested concentrations. From the absorbance measured in the test media in different treatments, was possible to determine decay of Ni at 96h (**Table 2).** With values around 4%, the maximal decay was 4.79% in 12.5 mg/l treatment. Decay could not be determined in the 100 mg/l treatment due to all fish were already dead by the 96h of experiment.

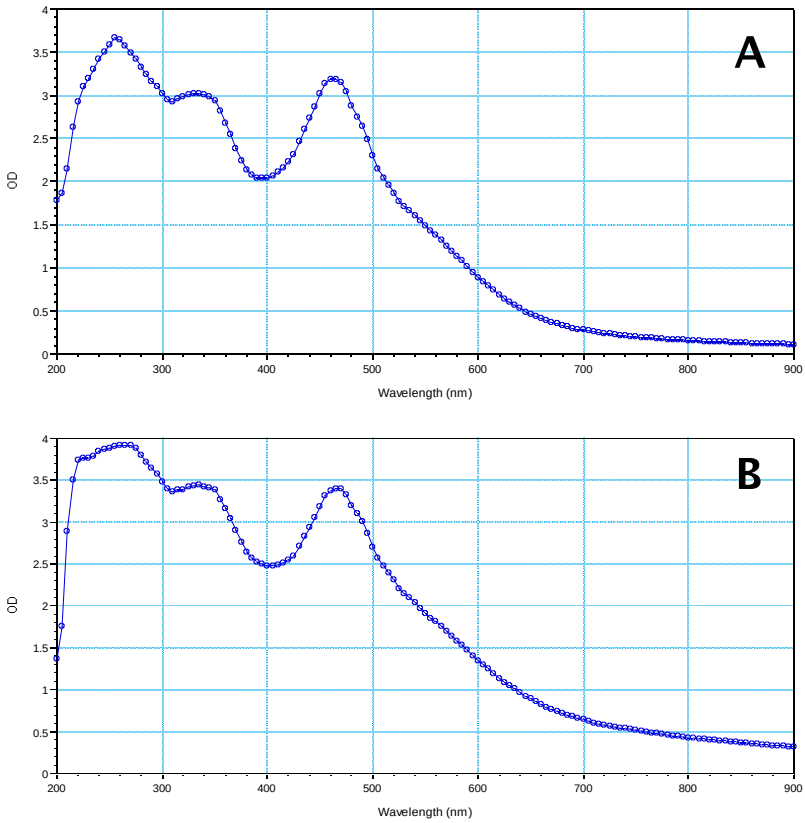

Figure 7 – Absorbance spectrums of a nickel solution (100 mg/l) prepared in u.p. water (**A**) and another in artificial salt water (**B**) showing a peak in the 470 nm.

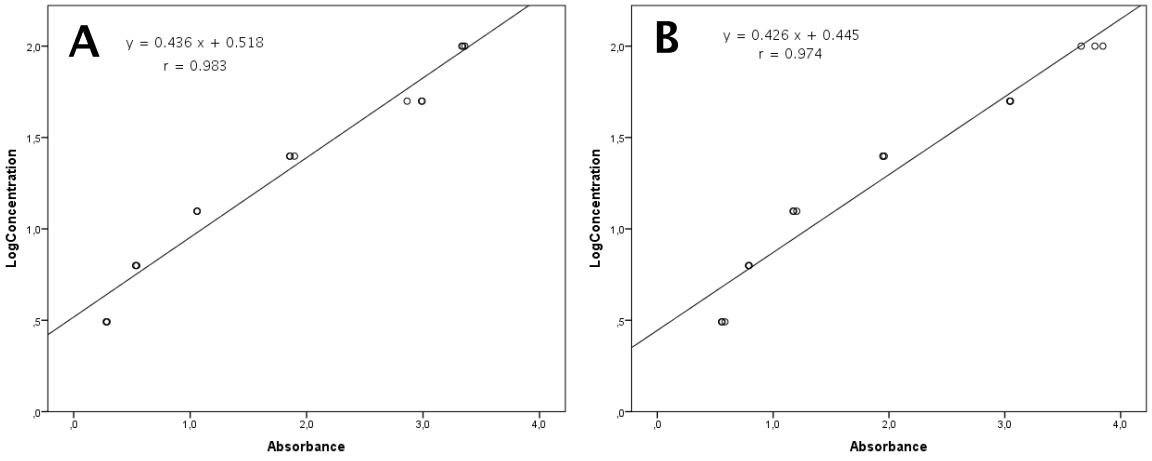

Figure 8 – Nickel standard curve in u.p. water (**A**) and artificial salt water (**B**) constructed by linear regression analysis of log of the concentration vs absorbance at 470 nm. r- correlation coefficient of Pearson.

Table 2 – Nickel actual concentrations in test media of the exposure bioassay of *Pomatoschistus microps* juveniles to nickel, calculated from absorbance readings at 470 nm and using the standard curve shown in **Figure 8B.** Values are the mean and corresponding standard errors of concentrations estimated for test solutions during the bioassay (N=3). Deviation - % of deviation between nominal and actual concentrations; Decay - % of nickel decay between 0h and 96h; "-" – All fish were dead.

Nickel concentration					
	0h		96h		
Nominal (mg/l)	**Actual** (mg/l)	**Deviation** (%)	**Actual** (mg/l)	**Deviation** (%)	**Decay** (%)
3.1	3.18 ± 0.01	2.58	3.12 ± 0.01	0.65	1.89
6.3	6.51 ± 0.21	3.33	6.23 ± 0.13	1.11	4.30
12.5	12.71 ± 0.38	1.68	12.10 ± 0.30	3.20	4.79
25.0	25.26 ± 0.72	1.04	24.23 ± 0.73	3.08	4.08
50.0	46.16 ± 0.47	7.68	44.00 ± 0.15	12.00	4.68
100.0	96.77 ± 1.15	3.23	-	-	-

3.2.3. Abiotic conditions and test organisms used

Table 3 and **4** indicate the values of abiotic parameters measured in test media during the exposure of fish from Minho and Lima river estuaries, respectively, to Ni. Regarding both estuaries, salinity remained constant in the 18 g/l and pH between 8.3 and 8.5 units. The temperature shows a variation range of about 2ºC. Dissolved oxygen was always above the 60% required by OECD as one of the criteria to valid a bioassay (OECD, 1992). In this bioassay sixty three randomly selected fish were used from each estuary, with an average standard length (mean ± SD) of 2.1 ± 0.2 cm from both estuaries, and average weight (mean ± SD) of 0.087 ± 0,022 g and 0.099 ± 0.038 g from Minho and Lima, respectively (**Table 5**).

Table 3 – Abiotic parameters measured in test media of the 96h bioassay carried out to investigate the effects of nickel on *Pomatoschistus microps* from the Minho river estuary. The determinations were made at the beginning (0h) and at each 24h. Values are the mean with the corresponding standard deviation (SD). DO – water dissolved oxygen. Ni – concentrations of nickel. "-" – All fish were dead.

Treatment (Ni mg/l)	**Exposure** (hours)	**Temperature** (ºC)	**Salinity** (g/l)	**pH**	**DO** (mg/l)
0	0h	18.24 ± 1.55	18 ± 1	8.35 ± 0.12	9.63 ± 0.38
	24h	18.14 ± 1.66		8.40 ± 0.07	9.64 ± 0.37
	48h	18.94 ± 0.92		8.41 ± 0.16	9.40 ± 0.10
	72h	18.36 ± 0.62		8.44 ± 0.15	9.46 ± 0.07
	96h	18.80 ± 1.05		8.42 ± 0.14	9.46 ± 0.12
	|96h-0h|	0.56 ± 0.50	0	0.07 ± 0.02	-0.17 ± 0.26
3.1	0h	17.90 ± 1.44	18 ± 1	8.39 ± 0.07	9.63 ± 0.38
	24h	17.81 ± 1.51		8.40 ± 0.05	9.64 ± 0.38
	48h	18.53 ± 0.80		8.43 ± 0.08	9.44 ± 0.14
	72h	18.08 ± 0.56		8.43 ± 0.09	9.48 ± 0.15
	96h	18.61 ± 0.98		8.43 ± 0.09	9.41 ± 0.19
	|96h-0h|	0.71 ± 0.46	0	0.04 ± 0.02	-0.22 ± 0.19
6.3	0h	17.83 ± 1.39	18 ± 1	8.38 ± 0.06	9.66 ± 0.35
	24h	17.43 ± 1.59		8.40 ± 0.04	9.73 ± 0.39
	48h	18.48 ± 1.06		8.42 ± 0.07	9.43 ± 0.18
	72h	17.81 ± 0.71		8.40 ± 0.07	9.38 ± 0.44
	96h	18.44 ± 1.01		8.42 ± 0.08	9.47 ± 0.20
	|96h-0h|	0.61 ± 0.38	0	0.04 ± 0.02	-0.19 ± 0.15
12.5	0h	18.18 ± 1.47	18 ± 1	8.38 ± 0.08	9.59 ± 0.38
	24h	17.93 ±1.47		8.39 ± 0.05	9.62 ± 0.36
	48h	18.82 ±0.96		8.41 ± 0.10	9.30 ± 0.32
	72h	18.33 ±0.54		8.44 ± 0.09	9.48 ± 0.10
	96h	18.67 ±1.00		8.43 ± 0.11	9.32 ± 0.36
	|96h-0h|	0.49 ± 0.47	0	0.15 ± 0.03	-0.27 ± 0.02
25.0	0h	18.09 ± 1.70	18 ± 1	8.37 ± 0.07	9.62 ± 0.41
	24h	17.69 ± 1.77		8.37 ± 0.04	9.60 ± 0.30
	48h	18.74 ± 1.40		8.41 ± 0.09	9.39 ± 0.29
	72h	17.88 ± 0.84		8.43 ± 0.09	9.54 ± 0.20
	96h	18.43 ± 1.56		8.45 ± 0.08	9.51 ± 0.34
	|96h-0h|	0.34 ± 0.14	0	0.08 ± 0.01	-0.11 ± 0.07
50.0	0h	18.19 ± 1.47	18 ± 1	8.36 ± 0.08	9.61 ± 0.37
	24h	17.96 ± 1.49		8.38 ± 0.05	9.63 ± 0.35
	48h	18.74 ± 0.89		8.40 ± 0.10	9.37 ± 0.21
	72h	18.16 ± 0.54		8.41 ± 0.11	9.48 ± 0.10
	96h	18.20 ± 0.98		8.46 ± 0.09	9.53 ± 0.20
	|96h-0h|	0.01 ± 0.49	0	0.10 ± 0.01	-0.08 ± 0.17
100	0h	17.99 ± 1.58	18 ± 1	8.35 ± 0.07	9.68 ± 0.39
	24h	17.29 ± 1.36		8.36 ± 0.05	9.75 ± 0.36
	48h	18.30 ± 1.32		8.40 ± 0.07	9.51 ± 0.26
	72h	17.37 ± 0.70		8.42 ± 0.11	9.70 ± 0.09
	96h	-		-	-
	|96h-0h|	-	-	-	-

Table 4 – Abiotic parameters measured in test media of the 96h bioassay carried out to investigate the effects of nickel on *Pomatoschistus microps* from the Lima river estuary. The determinations were made at the beginning (0h) and at each 24h. Values are the mean with the corresponding standard deviation (SD). DO – water dissolved oxygen. Ni – concentrations of nickel. "-" – All fish were dead.

Treatment (Ni mg/l)	**Exposure** (hours)	**Temperature** (ºC)	**Salinity** (g/l)	**pH**	**DO** (mg/l)
0	0h	17.42 ± 1.38	18 ± 1	8.40 ± 0.09	9.80 ± 0.27
	24h	17.08 ± 1.54		8.42 ± 0.06	9.86 ± 0.28
	48h	18.62 ± 1.28		8.43 ± 0.06	9.43 ± 0.23
	72h	17.77 ± 0.61		8.40 ± 0.14	9.51 ± 0.17
	96h	18.33 ± 1.43		8.40 ± 0.15	9.45 ± 0.39
	|96h-0h|	0.91 ± 0.05	0	0 ± 0.06	-0.35 ± 0.12
3.1	0h	17.30 ± 1.17	18 ± 1	8.45 ± 0.02	9.99 ± 0.53
	24h	17.18 ± 1.15		8.44 ± 0.02	9.83 ± 0.24
	48h	18.62 ± 0.96		8.46 ± 0.03	9.43 ± 0.14
	72h	17.95 ± 0.40		8.47 ± 0.06	9.55 ± 0.10
	96h	18.55 ± 1.17		8.48 ± 0.04	9.40 ± 0.30
	|96h-0h|	1.25 ± 0	0	0.03 ± 0.02	-0.59 ± 0.23
6.3	0h	17.38 ± 1.59	18 ± 1	8.46 ± 0.02	9.78 ± 0.42
	24h	16.98 ± 1.55		8.44 ± 0.02	9.80 ± 0.30
	48h	18.55 ± 1.41		8.45 ± 0.07	9.42 ± 0.28
	72h	17.92 ± 0.71		8.45 ± 0.07	9.56 ± 0.09
	96h	18.44 ± 1.51		8.46 ± 0.08	9.43 ± 0.40
	|96h-0h|	1.06 ± 0.08	0	0 ± 0.06	-0.35 ± 0.02
12.5	0h	17.38 ± 1.50	18 ± 1	8.45 ± 0.01	9.82 ± 0.33
	24h	17.03 ±1.26		8.44 ± 0.01	9.85 ± 0.26
	48h	18.62 ± 1.21		8.43 ± 0.05	9.40 ± 0.25
	72h	17.82 ±0.58		8.46 ± 0.06	9.59 ± 0.08
	96h	18.16 ±1.50		8.47 ± 0.04	9.50 ± 0.40
	|96h-0h|	0.78 ±0	0	0.02 ± 0.03	-0.32 ± 0.07
25.0	0h	17.67 ± 1.70	18 ± 1	8.44 ± 0.01	9.73 ± 0.37
	24h	17.30 ± 1.52		8.43 ± 0.03	9.76 ± 0.34
	48h	18.92 ± 1.53		8.47 ± 0.03	9.26 ± 0.41
	72h	17.55 ± 0.52		8.46 ± 0.04	9.47 ± 0.28
	96h	18.47 ± 1.52		8.44 ± 0.08	9.38 ± 0.44
	|96h-0h|	0.80 ± 0.18	0	0 ± 0.07	-0.35 ± 0.07
50.0	0h	17.42 ± 1.47	18 ± 1	8.43 ± 0.02	9.82 ± 0.32
	24h	17.08 ± 1.39		8.45 ± 0.03	9.86 ± 0.28
	48h	18.60 ± 1.19		8.47 ± 0.02	9.39 ± 0.27
	72h	17.80 ± 0.62		8.44 ± 0.07	9.56 ± 0.14
	96h	17.20 ± 0.26		8.48 ± 0.01	9.62 ± 0.28
	|96h-0h|	-0.22 ± 1.21	0	0.05 ± 0.01	-0.20 ± 0.04
100	0h	17.58 ± 1.74	18 ± 1	8.42 ± 0.10	9.75 ± 0.43
	24h	17.40 ± 1.33		8.44 ± 0.02	9.70 ± 0.41
	48h	18.72 ± 1.08		8.40 ± 0.07	9.41 ± 0.19
	72h	17.72 ± 0.50		8.40 ± 0.10	9.52 ± 0.13
	96h	-		-	-
	|96h-0h|	-	-	-	-

Table 5 – Measures of weight and length of the test organisms used in the 96h bioassay carried out to investigate the effects of nickel on *Pomatoschistus microps* from Minho and Lima river estuaries. Ni – concentrations of nickel.

	Minho Estuary		Lima Estuary	
Treatment (Ni mg/l)	**Weight** (g)	**Length** (cm)	**Weight** (g)	**Length** (cm)
0	0.0671	1.8	0.0688	1.9
	0.0663	1.8	0.0697	1.9
	0.0886	2.0	0.0845	2.1
	0.0789	2.1	0.1558	2.3
	0.0519	2.1	0.1651	2.4
	0.0699	2.0	0.1337	2.2
	0.1354	2.4	0.0894	2.1
	0.1277	2.2	0.0751	1.9
	0.1112	2.2	0.0598	1.8
3.1	0.0704	1.8	0.0629	1.9
	0.0621	1.8	0.0704	1.8
	0.0366	1.8	0.1560	2.4
	0.1163	2.3	0.1332	2.4
	0.0993	2.1	0.1268	2.3
	0.1038	2.2	0.0757	2.0
	0.1092	2.2	0.0622	1.8
	0.1118	2.2	0.0709	2.1
	0.1254	2.3		
6.3	0.0752	1.9	0.0435	1.8
	0.0487	1.8	0.0365	1.7
	0.0964	2.0	0.0943	2.1
	0.0955	2.1	0.1600	2.5
	0.0945	2.1	0.1478	2.4
	0.0873	2.0	0.1670	2.5
	0.0960	2.3		
	0.0939	2.2		
12.5	0.0767	2.0	0.1678	2.5
	0.0653	1.9	0.1116	2.2
	0.0588	1.8	0.1161	2.2
	0.1031	2.2	0.0671	1.9
	0.0921	2.0	0.0878	2.0
	0.0886	2.0	0.0896	2.1
	0.922	1.9		
	0.0909	2.0		
	0.0985	2.2		
25.0	0.0911	2.1	0.0810	2.0
	0.826	2.1	0.1381	2.0
	0.0916	2.1	0.0609	1.9
	0.0664	1.9	0.1535	2.3
	0.0881	2.1	0.1074	2.2
	0.0999	2.3	0.1156	2.2
			0.0816	2.1
			0.0945	2.1
50.0	0.1307	2.4	0.0563	1.8
	0.0531	1.8	0.0975	2.1
	0.0774	1.9	0.0975	2.2
	0.0711	2.0	0.0730	2.0
	0.0771	1.9	0.0594	2.0
	0.087 ± 0.022	2.1 ± 0.2	0.099 ± 0.038	2.1 ± 0.2

3.2.4. Mortality

As indicated in **Table 7**, along the 96h bioassay no mortality was recorded on the control group of both estuaries, being in agreement to OECD (1992) regulation. In the range of Ni concentrations tested (3.1mg/l to 100 mg/l) the lowest concentration causing mortality in *P. microps* from Lima was 3.1 mg/l (11.1% - 96h) whereas in *P. microps* from Minho was 6.3 mg/l (11.1% - 24h). Total mortality (100%) was observed in the treatment of 100 mg/l for both populations (96h). Some fish of this treatment were noticed to swim upside down some time before their deaths, indicating Ni intoxication (Scevevicius, 2010). Regarding total cumulative mortality in the end of the bioassay, in fish from Lima was slightly higher (33.3%) than in fish from Minho (26.9%). Determined from the deaths recorded after the 96h exposure, the LC_{50} values were similar for *P. microps* of both estuaries (**Table 6**). Comparing the LC_{50} obtained in the bioassay, 46.721mg/l and 44.025 mg/l for Minho and Lima populations respectively, with other European freshwater fish species, *P. microps* can be considered less sensitive to Ni than the rainbow trout (*Oncorhynchus mykiss*) (LC_{50} -19.3 mg/l) and the three-spined stickleback (*Gasterosteus aculeatus*) (LC_{50} -33.7 mg/l), as sensitive as Perch (*Perca fluviatilis*) (LC_{50} – 48.1 mg/l) and the roach (*Rutilus rutilus*) (LC_{50} – 48.7 mg/l), and more sensitive than the dace (*Leuciscus leuciscus*) (LC_{50} – 61.2 mg/l) (Svecevicius, 2010). Differences in susceptibility between different fish are related to physiological and metabolic activities of each species (Svecevicius, 2010).

These results suggest that juveniles from different populations have similar sensitivity to Ni, despite their different background of exposure to environmental contaminants.

Table 6 – Median lethal concentration (LC_{50}) in *Pomatoschistus microps* juveniles from Minho and Lima river estuaries from the 96h bioassay carried out to investigate the effects of nickel. 95%CL – 95% confidence limits.

LC_{50} (95%CI)	
Minho	**Lima**
46.721 (35.365-68.622)	44.025 (30.571-69.866)

Table 7 - Mortality along the 96h bioassay carried out to investigate the effects of nickel on *Pomatoschistus microps* juveniles from Minho and Lima river estuaries. Exposure to different experimental treatments: 3.1, 6.3, 12.5, 25.0, 50.0 and 100 mg/l of nickel. No mortality was recorded in the control groups of both estuaries. Ni – concentrations of nickel. "-" – no mortality.

	Mortality (%)							
	Minho Estuary				Lima Estuary			
Treatment (Ni mg/l)	**24h**	**48h**	**72h**	**96h**	**24h**	**48h**	**72h**	**96h**
0	-	-	-	-	-	-	-	-
3.1	-	-	-	-	-	-	-	11.1
6.3	11.1	11.1	11.1	11.1	11.1	11.1	33.3	33.3
12.5	-	-	-	-	-	-	11.1	33.3
25.0	11.1	11.1	11.1	33.3	-	-	-	11.1
50.0	-	11.1	11.1	44.4	11.1	22.2	33.3	44.4
100	33.3	66.6	77.7	100	11.1	33.3	66.6	100

3.2.5. Post-exposure predatory performance

Behavior can be considered as the expression of the health status of an individual (physiological functions) (Scott & Sloman, 2004), and thus, an important endpoint in environmental pollution effects research (Atchison et al., 1987). The predatory performance or just the capability to chase and ingest food, evaluated here as the percentage of prey (*Artemia franciscana* nauplii) ingested by fish, is vital not only to individual survival, but most important to the population level. A reduction in the predation efficiency leads to low levels of energy, necessary to growth and other functions like locomotion or reproduction (Little & Finger, 1990).

During the bioassay, some of the fish exposed to the treatments with higher concentrations of nickel (25.0, 50.0 and 100 mg/l) were observed swimming more to the surface than usual after 48h of exposure, showing first signs of nickel intoxication according to Svecevicius (2010). The post-exposure predatory performance test was performed in fish from all treatments, except in the 100 mg/l of Ni treatment where all fish were already dead by the end of the bioassay. **Figure 9A** illustrates the capability to ingest prey after the 96h exposure to Ni, showing values between 60% and 71% of offered prey ingestion in the control groups. No significant differences were found between populations from different

estuaries, yet are indicated significant differences between treatments (**Table 8**). Relatively to the control group, significant reduction of prey ingestion were observed in fish exposed to the higher concentrations, 12.5, 25.0 and 50.0 mg/l of Ni with inhibition values (**Figure 9B**) of 61%, 79% and 94%, respectively, in fish from Minho. Regarding fish from Lima inhibition values are around 30/37%, only significantly increasing in 50 mg/l of Ni treatment with 92% of inhibition. Origin do not interfered in the post-exposure predatory performance of the populations of *P. microps*. **Table 9** indicates similar EC_{50} values after the exposure to Ni, 15.618 mg/l for Minho population and 25.936 mg/l for Lima population.

Despite *P. microps* from population from Minho presented higher levels of inhibition at lower concentrations of Ni comparing to Lima population, showing to experience earlier the effects of Ni, results indicate a great increase of inhibition, almost total, in the 50.0 mg/l of Ni treatment in both populations.

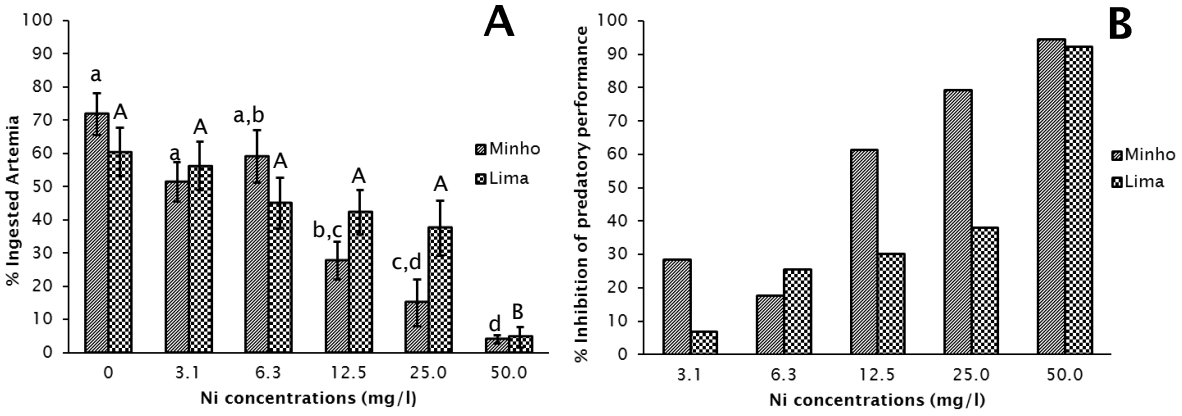

Figure 9 – Post-exposure predatory performance of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries after 96h of exposure to artificial salt water only (0) or to different nickel (Ni) concentrations. The predatory performance is expressed as the percentage of prey (*Artemia franciscana* nauplii) ingested by fish relatively to the control group (0) (**A**) and percentage of inhibition of prey ingestion of fish relatively to the control group (0) (**B**). The results are expressed as the mean of nine fish of each estuary per treatment with the corresponding standard error bars. Lower case and capital letters - Significant differences among treatments of Minho and Lima river estuaries, respectively, as indicated by one-way analysis of variance and the Turkey's test ($p \leq 0.05$).

Table 8 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for Post-exposure predatory performance of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the effects of nickel. Exposure to different experimental treatments: 0, 3.1, 6.3, 12.5, 25.0 and 50.0 mg/l of nickel. SEM – standard error of the mean. F – degrees of freedom. P – significance.

Comparison		Mean ± SEM	ANOVA	Tukey
Origin	Minho Lima	36.406 ± 1.840 38.230 ± 1.916	$F_{(1,87)} = 0.471$ $P > 0.05$	No significant differences
Treatment	**0**	55,470 ± 2.865	$F_{(5,87)} = 23.033$ $P \leq 0.05$	**a**
	3.1	47.548 ± 2.953		**a, b**
	6.3	46.589 ±3.282		**a, b**
	12.5	35.618 ± 3.203		**b, c**
	25.0	29.071 ± 3.282		**c**
	50.0	9.610 ± 3.844		**d**
Interaction	Origin*Treatment		$F_{(5,87)} = 2.284$ $P > 0.05$	No significant differences

Table 9 – Half maximal effective concentration (EC_{50}) in *Pomatoschistus microps* juveniles from Minho and Lima river estuaries from the 96h bioassay carried out to investigate the effects of nickel. 95%CL – 95% confidence limits.

$EC_{50(95\%CI)}$	
Minho	**Lima**
15.618 (3.281-46.971)	25.936 (17.577-42.276)

3.2.6. Analysis of biomarkers

The determination of biomarkers was performed in fish from all treatments, except in the 100 mg/l of Ni treatment where all fish were already dead by the end of the bioassay. Regarding AChE activity (**Figure 10A**), no significant differences were found between treatments. However, the population origin presented significant differences, with fish from Minho with a higher activity of this enzyme than fish from Lima (91.257 and 75.694 nmol /min /mg protein, respectively) (**Table 10**). The positive interaction between the two main factors suggests that the origin of the fish (estuary) influences the AChE activity determined in fish exposed to different treatments. Thus, long term exposure to environmental contamination during pre-development phases may have decreased the AChE activity of Lima estuary fish, suggesting that they were exposed to anticholinesterase agents. In relation to GST activity (**Figure 10C**), significant differences were found between the populations, with fish from Minho with lower values of activity of this enzyme than Lima (28.025 and 36.084 nmol/ min/ mg protein, respectively) (**Table 10**). Treatments indicate significant differences as well, showing an increase of GST activity as concentrations of Ni increases. From 22 nmol/ min/ mg protein in the control groups, to 46 nmol/min/ mg protein in treatments of 25.0 and 50.0 mg/l of Ni, about twice. High levels of Ni can produce ROS, which in high concentrations can cause oxidative stress, inducing the cellular antioxidant defense system, and so the GST activity, to prevent damage by enabling the excretion of the toxic compounds (Denkaus and Salniow, 2002; Sáenz et al., 2010). In agreement with these results, Mohammed (2014) and Wang & Wang (2010) found the same tendency of increase of the GST activity with the increase of Ni concentrations in copepods, *Apocyclops borneoensis* and *Tigriopus japonicus*, respectively. Interaction between the two main factors was not significative, suggesting that the origin of the fish (estuary) do not influences the GST activity determined in fish exposed to different treatments. EROD activity (**Figure 10B**) do not presented significant differences either between different populations or between the treatments tested. The interaction of the two factors also showed no significant differences (**Table 11**). This lack of results may be due to this particular technique is not yet optimized for this particular fish species. The *Pomatoschistus microps* is a small fish, and perhaps should have been utilized larger pools of fish content. Concerning the lipid peroxidation levels (LPO) (**Figure 10D**), significant

differences can be observed between estuaries, with population of Minho presenting higher oxidative stress than population from Lima (1.320 and 0.988 nmol TBARS/ mg protein, respectively) (**Table 11**). These results can be related to the higher GST activity in population of Lima mentioned above (**Table 10**), suggesting a better functioning of the cellular antioxidant defences in fish from Lima and lower levels of oxidative damage. Among treatments were also found significant differences, with induction of an increase of lipid peroxidation in treatments with the higher concentrations. Treatment of 25.0 mg/l on Ni in the population from Lima and both treatments of 25.0 and 50.0 mg/l of Ni in the population from Minho. Heavy metals are known to cause cell membrane damage by inducing lipid peroxidation mechanisms (Moahmmed, 2014).

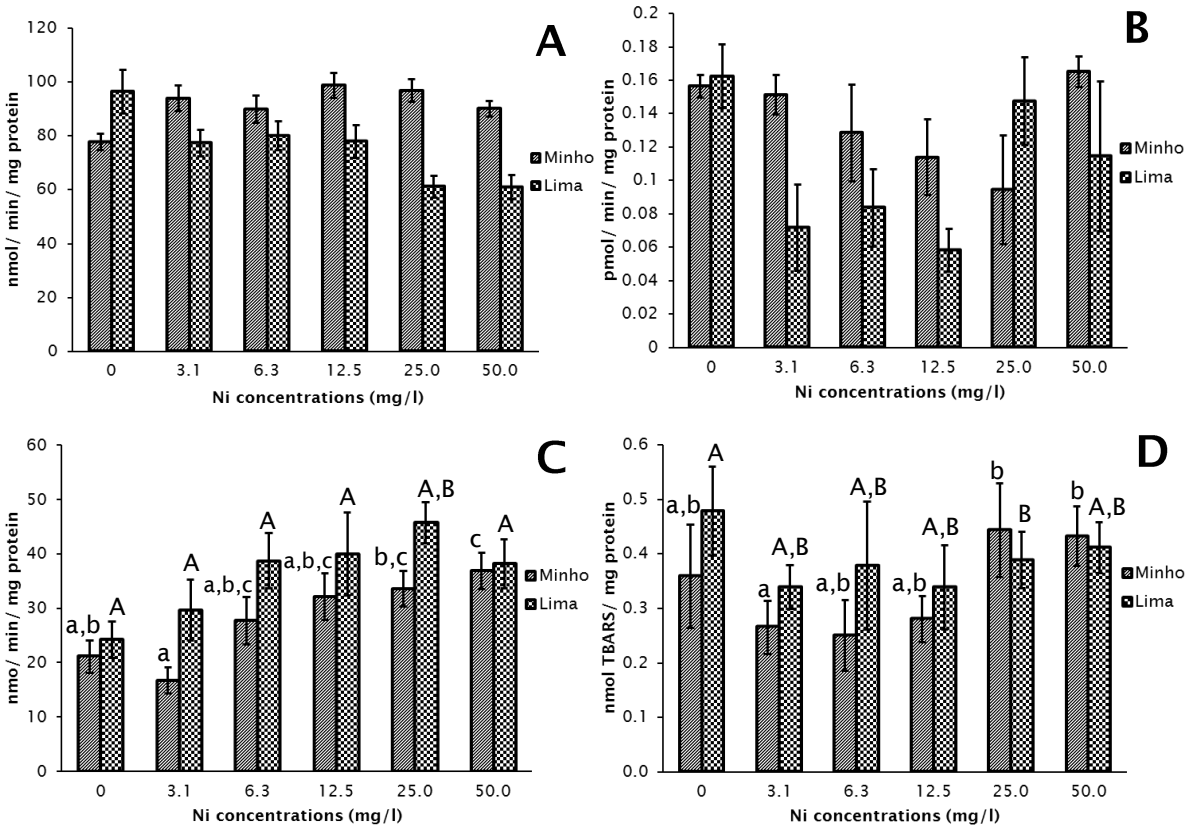

Figure 10 – Acetylcholinesterase (**A**), Glutathione S-tranferase (**C**) and ethoxyresorufin *O*-deethylase (**B**) activities and Lipid peroxidation levels (**D**) of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries after 96h of exposure to artificial salt water (0 – control group) only and to different concentration of nickel (Ni). The values are the mean of nine fish of each estuary per treatment with corresponding standard error bars. Lower case and capital letters - Significant differences among treatments of Minho and Lima river estuaries, respectively, as indicated by one-way analysis of variance and the Turkey's test ($p \leq 0.05$).

Table 10 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for Acetylcholinesterase (AChE) and Glutathione S-tranferase (GST) activities of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the effects of nickel. Exposure to different experimental treatments: 0, 3.1, 6.3, 12.5, 25.0 and 50.0 mg/l of nickel. SEM – standard error of the mean. F – degrees of freedom. P – Significance.

Biological Parameter	Comparison		Mean ± SEM	ANOVA	Tukey
AChE	**Origin**	Minho	91.257 ± 2.360	$F_{(1, 87)} = 20.868$	**a**
		Lima	75.694 ± 2.457	$P \leq 0.05$	**b**
	Treatment	0	87.136 ± 3.674	$F_{(5, 87)} = 1.298$ $P > 0.05$	No significant differences
		3.1	85.731 ± 3.787		
		6.3	85.090 ± 4.209		
		12.5	88.364 ± 4.108		
		25.0	79.022 ± 4.209		
		50.0	75.510 ± 4.930		
	Interaction	Origin Treatment		$F_{(5, 87)} = 5.975$ $P \leq 0.05$	**Significant differences**
GST	**Origin**	Minho	28.025 ± 1.757	$F_{(1, 87)} = 10.097$	**a**
		Lima	36.084 ± 1.829	$P \leq 0.05$	**b**
	Treatment	0	22.673 ± 2.763	$F_{(5, 87)} = 6.192$ $P \leq 0.05$	**a**
		3.1	23.199 ± 2.820		**a**
		6.3	33.109 ± 3.134		**a, b**
		12.5	36.052 ± 3.058		**a, b**
		25.0	39.672 ± 3.134		**b**
		50.0	37.522 ± 3.670		**b**
	Interaction	Origin Treatment		$F_{(5, 87)} = 0.611$ $P > 0.05$	No significant differences

Table 11 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for ethoxyresorufin *O*-deethylase (EROD) activity and Lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the effects of nickel. Exposure to different experimental treatments: 0, 3.1, 6.3, 12.5, 25.0 and 50.0 mg/l of nickel. SEM – standard error of the mean. F – degrees of freedom. P – Significance.

EROD	**Origin**	Minho Lima	0.135 ± 0.017 0.107 ± 0.014	$F_{(1, 36)}$ = 1.567 P > 0.05	No significant differences
	Treatment	0 3.1 6.3 12.5 25.0 50.0	0.162 ± 0.025 0.111 ± 0.025 0.106 ± 0.027 0.086 ± 0.027 0.121 ± 0.028 0.140 ± 0.030	$F_{(5, 36)}$ = 1.048 P > 0.05	No significant differences
	Interaction	Origin Treatment		$F_{(5, 36)}$ = 0.840 P > 0.05	No significant differences
LPO	**Origin**	Minho Lima	1.320 ± 0.081 0.988 ± 0.084	$F_{(1, 87)}$ = 8.098 P ≤ 0.05	**a** **b**
	Treatment	0 3.1 6.3 12.5 25.0 50.0	1.555 ± 0.126 1.611 ± 0.129 1.132 ± 0.144 0.964 ± 0.140 0.817 ± 0.144 0.844 ± 0.168	$F_{(5, 87)}$ = 6.528 P ≤ 0.05	**a, b** **a** **a, b, c** **b, c** **c** **c**
	Interaction	Origin Treatment		$F_{(5, 87)}$ = 1.109 P > 0.05	No significant differences

3.3. Influence of microplastics on the toxicity of nickel to juvenile fish from the Minho and Lima estuaries

3.3.1. Measurement of nickel and microplastics concentrations and its potential decay in the bioassay

As mentioned in section 3.2.1, nominal concentrations of the test substance must be confirmed (actual concentration) and potential decay evaluated in the bioassay. **Table 12** indicates the actual Ni concentrations determined in test media, calculated from the linear regression equation shown in **Figure 8B.** The maximal deviation calculated was 6.8%, in the treatment with 12.5 mg/l of Ni, so, according to OECD (1992) regulation, nominal concentrations are suitable to indicate the tested concentrations. The Measurement of absorbance in test media in different treatments allowed estimating decay of Ni at the end of the bioassay (**Table 12**). Decay values ranged from 0.6%/1.8% at treatments with lower concentrations to a maximum of 6.7% in the treatment with 12.5 mg/l of Ni. Regarding the MP, **Table 13** indicates the actual concentrations estimated in test media, calculated from a linear regression equation (y=58.862x + 11.177) from a standard curve previous performed by the team in the scope of the "SIGNAL" project. Actual concentrations have maximal deviation of 2% (treatments with MP only and 12.5 mg/l of Ni) allowing the use of nominal concentrations. At the end of the bioassay, were observed values of decay between 0.5% and 1.6%. Decay of both Ni and MP could not be determined in the 100 mg/l treatment due to all fish were already dead by the 96h of experiment.

3.3.2. Abiotic conditions and test organisms used

The measurement of abiotic parameters in test media during the exposure of fish from Minho and Lima to Ni and MP are indicated in **Table 14** and **15**, respectively. For both estuaries, salinity values remained constant in the 18 g/l and pH between 8.3 and 8.5 units. Slightly lower in test media with fish from Minho, temperature variation were again less than 2ºC. Dissolved oxygen was always above the 60% required by OECD as one of the criteria to valid a bioassay (OECD, 1992). In this bioassay Seventy two randomly selected fish were used from each estuary. Fish from Minho present an average standard length (mean ±

SD) of 2 ± 0.2 cm and average weight (mean ± SD) of 0.075 ± 0.020 g and fish from Lima 2.2 ± 0.2 cm and 0.106 ± 0.035 g (**Table 16**).

Table 12 – Nickel actual concentrations in test media of the exposure bioassay of *Pomatoschistus microps* juveniles to nickel with 0.184 mg/l of microplastics, calculated from absorbance readings at 470 nm and using the standard curve shown in **Figure 8B.** Values are the mean and corresponding standard errors of concentrations estimated for test solutions during the bioassay (N=3). Deviation - % of deviation between nominal and actual concentrations; Decay - % of nickel decay between 0h and 96h; "-" – All fish were dead.

Nickel concentration					
	0h		96h		
Nominal (mg/l)	**Actual** (mg/l)	**Deviation** (%)	**Actual** (mg/l)	**Deviation** (%)	**Decay** (%)
3.1	3.16 ± 0.01	1.94	3.14 ± 0.01	1.29	0.63
6.3	6.22 ± 0.01	1.27	6.11 ± 0.02	3.02	1.77
12.5	13.35 ± 0.48	6.80	12.45 ± 0.43	0.40	6.74
25.0	25.01 ± 0.24	0.04	24.18 ± 0.28	3.28	3.72
50.0	50.54 ± 1.72	1.08	49.01 ± 1.66	1.98	3.03
100.0	101.82 ± 1.82	1.82	-	-	-

Table 13 – Microplastics (MP) actual concentrations in test media of the exposure bioassay of *Pomatoschistus microps* juveniles to nickel with 0.184 mg/l of MP, calculated using a standard curve previously performed by the team in scope of the project "SIGNAL" (y=58.862x + 11.177). Values are the mean and corresponding standard errors of concentrations estimated for test solutions during the bioassay (N=3). Corresponding nickel treatment within brackets. Deviation - % of deviation between nominal and actual concentrations; Decay - % of MP decay between 0h and 96h; "-" – All fish were dead.

MP concentration					
	0h		96h		
Nominal (mg/l)	**Actual** (mg/l)	**Deviation** (%)	**Actual** (mg/l)	**Deviation** (%)	**Decay** (%)
0.184 (0)	0.188 ± 0.004	2.17	0.186 ± 0.004	1.09	1.06
0.184 (3.1)	0.186 ± 0.002	1.09	0.184 ± 0.002	0	1.07
0.184 (6.3)	0.187 ± 0.001	1.63	0.185 ± 0.002	0.54	1.07
0.184 (12.5)	0.188 ± 0.005	2.17	0.187 ± 0.004	1.09	0.53
0.184 (25.0)	0.185 ± 0.002	0.54	0.182 ± 0.003	1.09	1.62
0.184 (50.0)	0.185 ± 0.003	0.54	0.184 ± 0.002	0	0.55
0.184 (100)	0.186 ± 0.004	1.09	-	-	-

Table 14 – Abiotic parameters measured in test media of the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on effects of nickel on *Pomatoschistus microps* from the Minho river estuary. The determinations were made at the beginning (0h) and at each 24h. Values are the mean with the corresponding standard deviation (SD). DO – water dissolved oxygen. Ni – concentrations of nickel. MP - microplastics "-" – All fish were dead.

Treatment (Ni mg/l + MP)	**Exposure** (hours)	**Temperature** (ºC)	**Salinity** (g/l)	**pH**	**DO** (mg/l)
0	0h	23.52 ± 0.38	18 ± 1	8.46 ± 0.01	9.53 ± 0.10
	24h	23.99 ± 0.50		8.47 ± 0.03	9.55 ± 0.24
	48h	23.48 ± 0.53		8.49 ± 0.04	9.42 ± 0.08
	72h	23.86 ± 0.44		8.48 ± 0.02	9.50 ± 0.11
	96h	24.43 ± 0.43		8.50 ± 0.04	9.40 ± 0.17
	|96h-0h|	0.91 ± 0.05	0	0.04 ± 0.03	-0.13 ± 0.07
MP	0h	23.61 ± 0.27	18 ± 1	8.46 ± 0.01	9.55 ± 0.28
	24h	23.91 ± 0.49		8.48 ± 0.04	9.59 ± 0.38
	48h	23.61 ± 0.49		8.49 ± 0.02	9.41 ± 0.25
	72h	23.79 ± 0.46		8.50 ± 0.01	9.50 ± 0.15
	96h	24.30 ± 0.56		8.50 ± 0.04	9.45 ± 0.20
	|96h-0h|	0.69 ± 0.29	0	0.04 ± 0.03	-0.10 ± 0.08
3.1+MP	0h	23.64 ± 0.30	18 ± 1	8.47 ± 0.01	9.56 ± 0.25
	24h	24.04 ± 0.32		8.49 ± 0.04	9.63 ± 0.20
	48h	23.59 ± 0.29		8.50 ± 0.03	9.50 ± 0.18
	72h	23.87 ± 0.30		8.51 ± 0.01	9.48 ± 0.34
	96h	24.31 ± 0.24		8.53 ± 0.01	9.47 ± 0.20
	|96h-0h|	0.67 ± 0.06	0	0.06 ± 0	-0.09 ± 0.05
6.3+MP	0h	23.54 ± 0.25	18 ± 1	8.46 ± 0.02	9.59 ± 0.38
	24h	23.90 ±0.31		8.50 ± 0.01	9.49 ± 0.36
	48h	23.49 ± 0.25		8.50 ± 0.01	9.35 ± 0.32
	72h	23.79 ± 0.25		8.49 ± 0.01	9.33 ± 0.10
	96h	24.29 ± 0.28		8.52 ± 0.02	9.40 ± 0.36
	|96h-0h|	0.75 ±0.03	0	0.06 ± 0	-0.19 ± 0.02
12.5+MP	0h	23.79 ± 0.22	18 ± 1	8.46 ± 0.01	9.62 ± 0.31
	24h	24.06 ± 0.24		8.50 ± 0.03	9.50 ± 0.20
	48h	23.57 ± 0.16		8.50 ± 0.01	9.40 ± 0.19
	72h	23.88 ± 0.16		8.48 ± 0.04	9.43 ± 0.20
	96h	24.39 ± 0.18		8.52 ± 0.01	9.57 ± 0.24
	|96h-0h|	0.60 ± 0.04	0	0.06 ± 0	-0.05 ± 0.07
25.0+MP	0h	23.72 ± 0.20	18 ± 1	8.45 ± 0.01	9.61 ± 0.27
	24h	23.96 ± 0.29		8.49 ± 0.03	9.53 ± 0.25
	48h	23.56 ± 0.25		8.49 ± 0.01	9.47 ± 0.11
	72h	23.91 ± 0.24		8.49 ± 0.01	9.58 ± 0.10
	96h	24.33 ± 0.20		8.52 ± 0.01	9.58 ± 0.20
	|96h-0h|	0.61 ± 0	0	0.07 ± 0	-0.03 ± 0.07
50.0+MP	0h	23.49 ± 0.28	18 ± 1	8.45 ± 0.02	9.60 ± 0.29
	24h	23.58 ± 0.15		8.50 ± 0.01	9.75 ± 0.26
	48h	23.63 ± 0.32		8.47 ± 0.02	9.58 ± 0.36
	72h	24.15 ± 0.21		8.48 ± 0	9.64 ± 0.10
	96h	24.7 ± 0		8.46 ± 0	9.58 ± 0.19
	|96h-0h|	1.21 ± 0.28	0	0.01 ± 0.02	-0.02 ± 0.10
100+MP	0h	23.61 ± 0.09	18 ± 1	8.43 ± 0.01	9.60 ± 0.26
	24h	23.85 ± 0.07		8.49 ± 0.01	9.51 ± 0.18
	48h	-		-	-
	72h	-		-	-
	96h	-		-	-
	|96h-0h|	-	-	-	-

Table 15 – Abiotic parameters measured in test media of the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on effects of nickel on *Pomatoschistus microps* from the Lima river estuary. The determinations were made at the beginning (0h) and at each 24h. Values are the mean with the corresponding standard deviation (SD). DO – water dissolved oxygen. Ni – concentrations of nickel. MP - microplastics "-" – All fish were dead.

Treatment (Ni mg/l + MP)	**Exposure** (hours)	**Temperature** (ºC)	**Salinity** (g/l)	**pH**	**DO** (mg/l)
0	0h	21.74 ± 0.29	18 ± 1	8.34 ± 0.06	9.76 ± 0.26
	24h	21.78 ± 0.22		8.37 ± 0.03	9.80 ± 0.27
	48h	21.87 ± 0.26		8.39 ± 0.01	9.84 ± 0.22
	72h	21.86 ± 0.31		8.36 ± 0.03	9.69 ± 0.18
	96h	21.90 ± 0.17		8.35 ± 0.04	9.75 ± 0.36
	|96h-0h|	0.16 ± 0.12	0	0.01 ± 0.02	-0.01 ± 0.10
MP	0h	21.83 ± 0.28	18 ± 1	8.43 ± 0.03	9.86 ± 0.33
	24h	21.57 ± 0.16		8.41 ± 0.02	9.89 ± 0.22
	48h	21.70 ± 0.21		8.42 ± 0.01	9.65 ± 0.24
	72h	21.66 ± 0.19		8.41 ± 0.02	9.76 ± 0.20
	96h	21.79 ± 0.18		8.41 ± 0.03	9.75 ± 0.30
	|96h-0h|	-0.04 ± 0.10	0	-0.02 ± 0	-0.11± 0.03
3.1+MP	0h	21.77 ± 0.25	18 ± 1	8.46 ± 0.01	9.78 ± 0.42
	24h	21.56 ± 0.36		8.40 ± 0.04	9.80 ± 0.30
	48h	21.64 ± 0.34		8.43 ± 0.03	9.52 ± 0.28
	72h	21.62 ± 0.26		8.43 ± 0.03	9.57 ± 0.20
	96h	21.60 ± 0.26		8.40 ± 0.03	9.50 ± 0.40
	|96h-0h|	-0.17 ± 0.01	0	-0.06 ± 0.02	-0.28 ± 0.02
6.3+MP	0h	21.89 ± 0.16	18 ± 1	8.45 ± 0.01	9.62 ± 0.33
	24h	21.60 ± 0.22		8.44 ± 0.02	9.75 ± 0.26
	48h	21.78 ± 0.23		8.41 ± 0.02	9.40 ± 0.30
	72h	21.97 ±0.16		8.42 ± 0.03	9.59 ± 0.10
	96h	21.90 ± 0.24		8.46 ± 0.03	9.50 ± 0.38
	|96h-0h|	0.01 ± 0.08	0	0.01 ± 0.02	-0.12 ± 0.05
12.5+MP	0h	21.92 ± 0.17	18 ± 1	8.43 ± 0.04	9.53 ± 0.29
	24h	21.86 ± 0.17		8.42 ± 0.02	9.76 ± 0.34
	48h	21.96 ± 0.15		8.43 ± 0.04	9.66 ± 0.27
	72h	21.93 ± 0.11		8.43 ± 0.03	9.47 ± 0.28
	96h	21.80 ± 0.20		8.41 ± 0.03	9.58 ± 0.38
	|96h-0h|	-0.12 ± 0.03	0	-0.02 ± 0.01	0.05 ± 0.09
25.0+MP	0h	21.98 ± 0.41	18 ± 1	8.42 ± 0.03	9.89 ± 0.32
	24h	22.04 ± 0.32		8.42 ± 0.02	9.81 ± 0.28
	48h	22.10 ± 0.35		8.43 ± 0.03	9.70 ± 0.27
	72h	22.20 ± 0.45		8.42 ± 0.03	9.56 ± 0.14
	96h	22.18 ± 0.26		8.42 ± 0.03	9.73 ± 0.28
	|96h-0h|	0.20 ± 0.15	0	0 ± 0	-0.16 ± 0.04
50.0+MP	0h	22.12 ± 0.27	18 ± 1	8.42 ± 0.02	9.65 ± 0.33
	24h	22.12 ± 0.36		8.40 ± 0.03	9.70 ± 0.31
	48h	22.19 ± 0.28		8.41 ± 0.04	9.51 ± 0.29
	72h	22.23 ± 0.27		8.40 ± 0.05	9.42 ± 0.13
	96h	22.15 ± 0.17		8.40 ± 0.06	9.63 ± 0.35
	|96h-0h|	0.03 ± 0.10	0	-0.02 ± 0.04	-0.02 ± 0.02
100+MP	0h	22.14 ± 0.25	18 ± 1	8.43 ± 0.02	9.55 ± 0.23
	24h	22.08 ± 0.40		8.42 ± 0.02	9.57 ± 0.18
	48h	22.09 ± 0.39		8.42 ± 0.02	9.64 ± 0.39
	72h	22.13 ± 0.15		8.41 ± 0.02	9.42 ± 0.23
	96h	-		-	-
	|96h-0h|	-	0	-	-

Table 16 – Measures of weight and length of the test organisms used in the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on the effects of nickel on *Pomatoschistus microps* from Minho and Lima river estuaries. Ni – concentrations of nickel. MP - microplastics

	Minho River Estuary		Lima River Estuary	
Treatment (Ni mg/l + MP)	**Weight** (g)	**Length** (cm)	**Weight** (g)	**Length** (cm)
0	0.0925	2.1	0.1278	2.3
	0.1324	2.5	0.1328	2.4
	0.1191	2.2	0.1215	2.2
	0.0813	2.1	0.1248	2.3
	0.0874	2.0	0.1088	2.1
	0.0679	1.9	0.1294	2.2
	0.0994	2.0	0.1792	2.6
	0.0884	2.1	0.1500	2.4
	0.1200	2.1	0.1661	2.4
MP	0.0755	1.9	0.1411	2.4
	0.0547	1.8	0.1661	2.4
	0.0655	1.9	0.2094	2.6
	0.0907	2.2	0.0635	1.9
	0.1026	2.2	0.0865	2.0
	0.0934	2.1	0.1042	2.2
	0.0668	1.8	0.0907	2.1
	0.0717	2.0	0.1699	2.4
			0.0888	2.1
3.1+MP	0.0756	2.0	0.1078	2.2
	0.0840	2.0	0.0831	2.0
	0.0760	2.0	0.0746	1.9
	0.0797	2.0	0.0653	2.0
	0.0485	1.9	0.0960	2.2
	0.0569	1.8	0.0942	2.1
	0.0707	1.9	0.1415	2.3
	0.0797	2.0	0.0490	1.7
	0.0679	2.0	0.0769	2.0
6.3+MP	0.0710	1.9	0.1434	2.4
	0.0801	2.0	0.0803	2.0
	0.0687	2.0	0.1706	2.5
	0.0804	1.9	0.1013	2.2
	0.0505	1.7	0.1130	2.2
	0.0557	1.8	0.1061	2.2
	0.0561	1.9	0.1024	2.1
	0.0551	1.9	0.1460	2.4
	0.0918	2.1	0.0485	1.9
12.5+MP	0.0611	1.9	0.0694	2.0
	0.0598	1.8	0.0958	2.2
	0.0504	1.8	0.1092	2.2
	0.0486	1.7	0.0956	2.0
	0.0698	2.0	0.0616	1.8
	0.0423	1.7	0.0651	2.0
	0.0746	2.0	0.1198	2.1
	0.0726	1.9	0.1205	2.2
	0.0979	2.1	0.1355	2.3
25.0+MP	0.0779	2.1	0.0836	2.0
	0.0641	1.9	0.0940	2.1
	0.0665	1.8	0.0848	2.0
	0.0687	1.9	0.0690	1.9
	0.0633	1.9	0.0682	2.0
	0.1079	2.2	0.0682	1.9
	0.0470	1.7	0.0843	2.2
			0.0852	1.9
			0.1025	2.2
50.0+MP	0.0551	1.8	0.1254	2.3
			0.1035	2.3
			0.0658	1.9
			0.1033	2.1
	0.075 ± 0.020	2.0 ± 0.2	0.106 ± 0.035	2.2 ± 0.2

3.3.4. Mortality

During the 96h of exposure to Ni and MP no mortality was recorded in fish from the control groups (**Table 17**), being once more according to OECD (1992) regulation. **Table 17** shows mortality results in the range of treatment tested, and due to equal MP concentrations in every treatment, from now on the reference will be only the Ni concentration of each treatment. In the treatment with MP alone was observed the death of one fish from Minho at 48h of exposure. Taking into account a previous study from Oliveira *et al*. (2013), who tested the same MP at the same concentrations not expecting to cause death alone and results supporting it, suggests than this isolated fish death incident was due to poor health prior to the bioassay. At the range of 3.1 to 100 mg/l of Ni tested, the lowest concentration causing mortality in *P. microps* from both Minho and Lima was 25.0 mg/l, with 22.2% and 11.1% mortality, respectively, at 96h. Total mortality (100%) was observed, once again, in the treatment of 100mg/l for both populations (96h). In this bioassay was also observed fish swimming upside down in this treatment as in the 100 mg/l of Ni alone of the previous bioassay (section 3.2.4), indicative of Ni intoxication (Scevevicius, 2010). Total cumulative mortality in the end of the bioassay was 27.8% in fish from Minho and 20.8% in fish from Lima. **Table 18** indicates the values of LC_{50}, determined from the deaths recorded after the 96h exposure, 34.698 mg/l in population from Minho and 47.080 mg/l in population from Lima.

These values are similar to those obtained in the bioassay of exposure to Ni alone (**Table 6**-section 3.2.4.). Comparing mortality results of the two bioassays (**Table 7**-section 3.2.4.), was observed an increase in the lower Ni concentration causing mortality (3.1/6.3 mg/l to 25mg/l), total mortality in 100 mg/l of Ni at 96h is maintained and total cumulative mortality remain similar (26.9% to 27.8% and 33.3% to 20.8% in Minho and Lima respectively). So, the presence of MP do not "cut" the toxic effects of Ni, however, seems to cause a "temporary protecting from death" effect, by delaying the influence of Ni in fish, suggesting toxicologically relevant interactions between MP and Ni. This may be due to some sort of adsorption mechanism of Ni to MP, with a potential threshold. This delay effect was already observed in relation to a PAH named pyrene (Oliveira *et al*., 2013).

Table 17 – Mortality along the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on the effects of nickel on *Pomatoschistus microps* juveniles from Minho and Lima river estuaries. Exposure to different experimental treatments: microplastics (MP) alone (0.184 mg/l) and six nickel (Ni) mixture treatments,100 mg/l of Ni + 0.184 mg/l of MP, 50.0 mg/l of Ni + 0.184 mg/l of MP, 25.0 mg/l of Ni + 0.184 mg/l of MP, 12.5 mg/l of Ni + 0.184 mg/l of MP, 6.3 mg/l of Ni + 0.184 mg/l of MP, and 3.1 mg/l of Ni + 0.184 mg/l of MP. No mortality was recorded in the control groups of both estuaries; "-" – no mortality. Ni – concentrations of nickel. MP – microplastics. "-" – no mortality.

	Mortality (%)							
	Minho Estuary				Lima Estuary			
Treatment (Ni mg/l + MP)	**24h**	**48h**	**72h**	**96h**	**24h**	**48h**	**72h**	**96h**
0	-	-	-	-	-	-	-	-
MP	-	11.1	11.1	11.1	-	-	-	-
3.1+MP	-	-	-	-	-	-	-	-
6.3+MP	-	-	-	-	-	-	-	-
12.5+MP	-	-	-	-	-	-	-	-
25.0+MP	-	-	-	22.2	-	-	-	11.1
50.0+MP	44.4	66.6	77.7	88.8	-	-	11.1	55.5
100+MP	77.7	88.8	88.8	100	-	11.1	66.6	100

Table 18 – Median lethal concentration (LC_{50}) in *Pomatoschistus microps* juveniles from Minho and Lima river estuaries from the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on the effects of nickel. 95%CL – 95% confidence limits.

LC_{50} (95%CI)	
Minho	**Lima**
34.698	47.080
(22.195-68.809)	(37.568-68.707)

3.3.5. Post-exposure predatory performance

As observed in the bioassay of exposure to Ni alone (section 3.2.5), some of the fish were detected swimming more to the surface than the rest, after 48h of exposure in treatments with 25.0, 50.0 and 100 mg/l of nickel (signs of Ni poisoning (Svecevicius, 2010)).

The post-exposure predatory performance test was performed in fish from all treatments, except in the 100 mg/l of Ni treatment where all fish were already dead by the end of the bioassay. **Figure 11A** indicates the results for the predatory performance, with between 87% and 93% of offered prey ingestion in the control groups. Due to equal MP concentrations in every treatment, from now on the reference will be only the Ni concentration of each treatment. Significant differences were found between treatments (**Table 19**), with significant reduction of prey ingestion observed in fish from both estuaries exposed to the treatments of MP alone and with the higher concentrations of Ni. Regarding the control group, fish from Minho exhibit significant inhibition values in treatments with 12.5, 25.0 and 50.0 mg/l of Ni with inhibition of 32%, 72% and total (100%), respectively. Fish from Lima show significant inhibition in more treatments, 6.3, 12.5, 25.0 and 50.0 mg/l of Ni, with 39%, 42%, 65% and 96% of reduction of prey ingestion, respectively. Regarding the treatment with MP alone is observed an inhibition of about 30% in both populations, perhaps due to ingestion of the MP, causing a feeling of satiety and leading to fish stops feeding themselves. Origin do not interfered in the predatory performance of the populations of *P. microps*. **Table 20** presents the values of EC_{50} of fish exposed to Ni and MP, with 20.119 mg/l for population from Minho 18.369 mg/l for population of Lima. These values are similar to the values obtained in the exposure to Ni alone bioassay (**Table 9**-section 3.2.5.).

In comparison, fish exposed to Ni with MP present a higher predatory performance relatively to fish exposed to Ni alone (section 3.2.5.), suggesting that the effects of Ni that remain after the exposure are attenuated by the presence of MP.

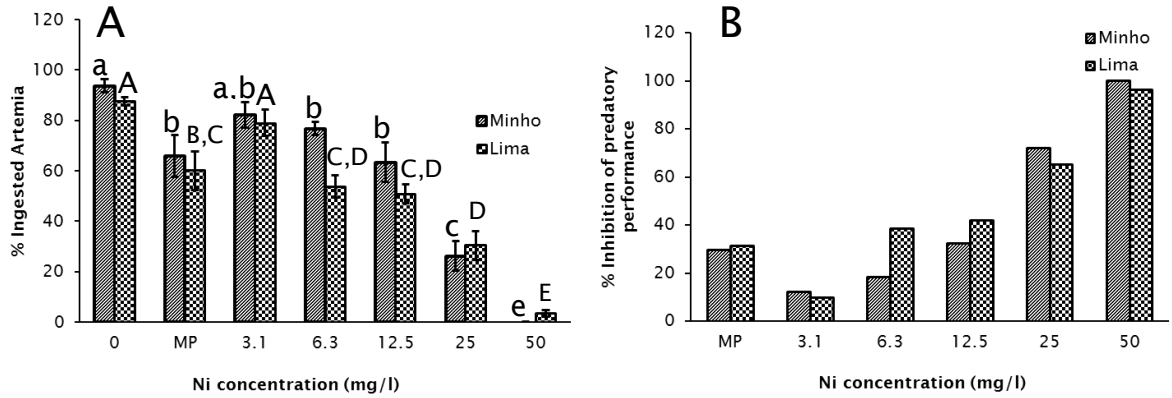

Figure 11 – Post-exposure predatory performance of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries after 96h of exposure to artificial salt water only (0), microplastics (0.184 mg/l) alone, and to different nickel concentrations with microplastics (0.184 mg/l). The predatory performance is expressed as the percentage of prey (*Artemia franciscana* nauplii) ingested by fish relatively to the control group (0) (**A**) and percentage of inhibition of prey ingestion of fish relatively to the control group (0) (**B**). The results are expressed as the mean of nine fish of each estuary per treatment with the corresponding standard error bars. Lower case and capital letters - Significant differences among treatments of Minho and Lima river estuaries, respectively, as indicated by one-way analysis of variance and the Turkey's test ($p \leq 0.05$). Ni – nickel concentration. MP – microplastics.

Table 19 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for Post-exposure predatory performance of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on the effects of nickel. Exposure to different experimental treatments: artificial salt water only (0), microplastics (0.184 mg/l) alone, and to different nickel concentrations with microplastics (0.184 mg/l). MP – microplastics. SEM – standard error of the mean. F – degrees of freedom. P – significance.

Comparison		Mean ± SEM	ANOVA	Tukey
Origin	Minho Lima	49.497 ± 2.028 45.502 ± 1.495	$F_{(1,87)} = 2.514$ $P > 0.05$	No significant differences
Treatment	**0**	74.147 ± 2.558	$F_{(5,87)} = 36.380$ $P \leq 0.05$	**a**
	MP	53.416 ± 2.636		**a, b**
	3.1	65.457 ± 2.558		**b, c**
	6.3	54.305 ± 2.558		**b, c**
	12.5	49.571 ± 2.558		**c**
	25.0	31.098 ± 2.808		**d**
	50.0	4.500 ± 6.066		**e**
Interaction	Origin*Treatment		$F_{(5,87)} = 1.210$ $P > 0.05$	No significant differences

Table 20 – Half maximal effective concentration (EC_{50}) in *Pomatoschistus microps* juveniles from Minho and Lima river estuaries from the 96h bioassay carried out to investigate the influence of microplastics in the effects of nickel. 95%CL – 95% confidence limits.

EC_{50} (95%CI)	
Minho	**Lima**
20.119	18.369
(14.225-33.181)	(10.188-34.862)

3.3.6. Analysis of Biomarkers

The determination of biomarkers was performed in fish from all treatments, except in the 100 mg/l of Ni treatment where, once again, all fish were already dead at the 96h of exposure. Concerning the AChE activity (**Figure 12A),** significante differences between estuaries were found, with fish from Minho presenting 97.776 nmol/ min/ mg protein and from Lima 57.295 nmol/ min/ mg protein (**Table 21**). Significant differences were found in treatments, with an incrising in AChE activity with the increase of the Ni concentrations. Heavy metals as shown to act as neurotoxic agents in aquatic organisms affecting AChE activity in other estudies (Forget *et al*., 1999; Wang & Wang, 2009), and Ni in particular as been reported to inhibit the AChE activity in copepod *T. brevicornis* (Mohammed, 2014). Therefore, these results can suggest some kind of interaction between MP and Ni. The positive interaction between the origin (estuaries) and the treatments suggests that the estuary influences the AChE activity determined in fish exposed to different treatments. So, long term exposure to environmental contamination during pre-development phases may have decreased the AChE of fish from Lima, suggesting the presence of anticholinesterase agents in Lima estuary. Regarding GST activity (**Figure 11C**), significant differences were found between the populations, with 37.796 nmol/ min/ mg protein and 24.088 nmol/ min/ mg protein from Minho and Lima populations (**Table 21**). In the treatments were follow the same tendency mentioned in section 3.2.6., with increase of GST activity with higher concentrations of Ni. The positive interaction between the two main factors suggests that the population origin influences the GST activity

determined in fish exposed to different treatments. EROD activity (**Figure 11B**) presented significant differences between fish of different estuaries, with the population of Minho showing higher activity values than population from Lima, with 0.070 pmol/ min/ mg protein and 0.024 pmol/ min/ mg protein, respectively. No significant differences between treatments or in the interaction of the two factors were found (**Table 22**). In relation to the lipid peroxidation (LPO) (**Figure 11D**), the origin of the fish populations do not represent an influence in their levels (**Table 22**). Significant differences can be observed between treatments, however the "up and down" along the increase of Ni concentrations seems weird, suggesting that something may went wrong in the procedure.

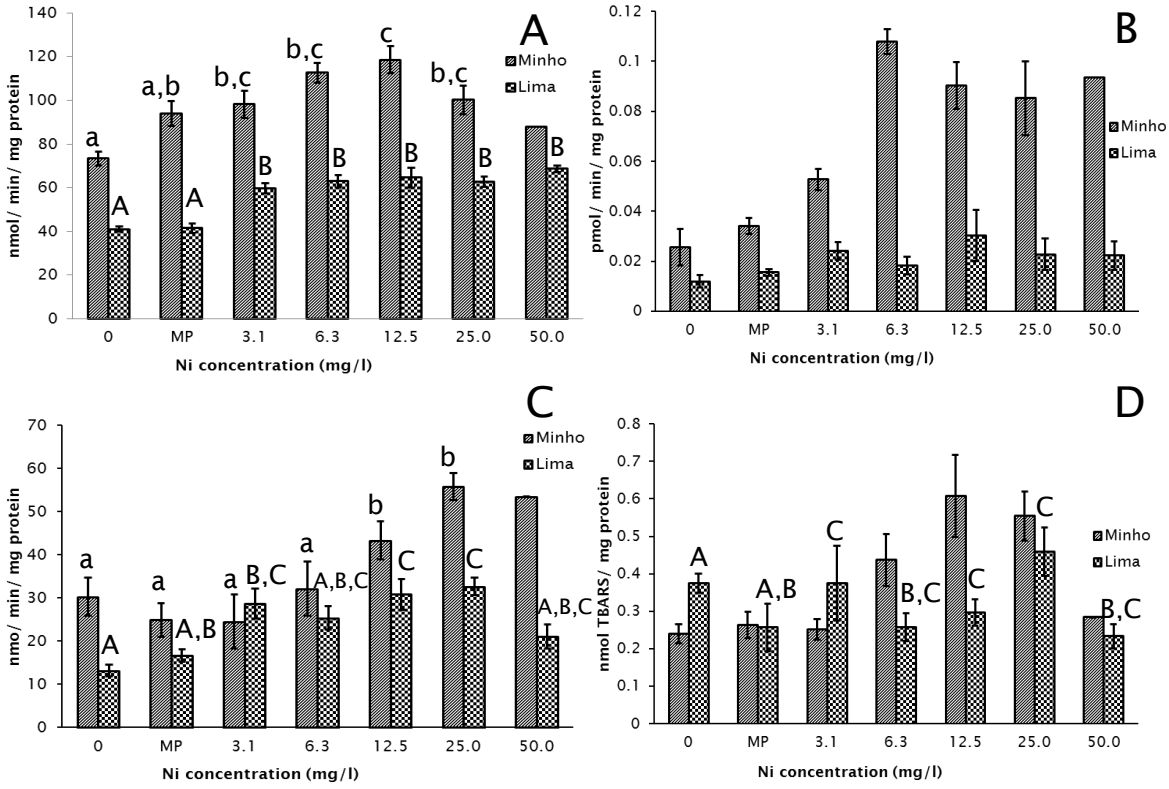

Figure 12 – Acetylcholinesterase (**A**), Glutathione S-tranferase (**C**) and ethoxyresorufin *O*-deethylase (**B**) activities and Lipid peroxidation levels (**D**) of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries after 96h of exposure to artificial salt water (0 – control group) only, microplastics (0.184 mg/l) alone and different concentration of nickel with microplastics (0.184 mg/l). The values are the mean of nine fish of each estuary per treatment with corresponding standard error bars. Lower case and capital letters - Significant differences among treatments of Minho and Lima river estuaries, respectively, as indicated by one-way analysis of variance and the Turkey's test ($p \leq 0.05$). Ni – nickel concentrations. MP – microplastics.

Table 21 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for Acetylcholinesterase (AChE) and Glutathione S-tranferase (GST) activities of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on effects of nickel. Exposure to different experimental treatments: artificial salt water only (0), microplastics (0.184 mg/l) alone, and to different nickel concentrations with microplastics (0.184 mg/l). MP – microplastics. SEM – standard error of the mean. F – degrees of freedom. P – significance.

Biological Parameter	Comparison		Mean ± SEM	ANOVA	Tukey
AChE	**Origin**	Minho	97.776 ± 2.323	$F_{(1, 87)}$ = 196.820	**a**
		Lima	57.295 ± 1.712	P ≤ 0.05	**b**
	Treatment	0	57.181 ± 2.928	$F_{(5, 87)}$ = 15.96 P ≤ 0.05	**a**
		MP	67.689 ± 3.019		**a, b**
		3.1	78.933 ± 2.928		**b, c**
		6.3	87.710 ± 2.928		**c**
		12.5	91.602 ± 2.928		**c**
		25.0	81.391 ± 3.215		**b, c**
		50.0	78.241 ± 6.945		**b**
	Interaction	Origin Treatment		$F_{(5, 87)}$ = 2.440 P ≤ 0.05	**Significant differences**
GST	**Origin**	Minho	37.796 ± 2.196	$F_{(1, 87)}$ = 25.257 P ≤ 0.05	**a**
		Lima	24.088 ± 1.619		**b**
	Treatment	0	21.758 ± 2.768	$F_{(5, 87)}$ = 8.440 P ≤ 0.05	**a**
		MP	20.816 ± 2.853		**a**
		3.1	26.628 ± 2.768		**a, b**
		6.3	28.786 ± 2.768		**a, b**
		12.5	37.097 ± 2.768		**b, c**
		25.0	44.248 ± 3.039		**c**
		50.0	37.264 ± 6.566		**a, b**
	Interaction	Origin Treatment		$F_{(5, 87)}$ = 2.716 P ≤ 0.05	Significant differences

Table 22 – Synthesis of two-way analysis of variance (ANOVA) and Tukey post hoc test for ethoxyresorufin *O*-deethylase (EROD) activity and Lipid peroxidation levels (LPO) of *Pomatoschistus microps* juveniles from Minho and Lima river estuaries (origin) after the 96h bioassay carried out to investigate the influence of microplastics (0.184 mg/l) on effects of nickel. Exposure to different experimental treatments: artificial salt water only (0), microplastics (0.184 mg/l) alone, and to different nickel concentrations with microplastics (0.184 mg/l). MP – microplastics. SEM – standard error of the mean. F – degrees of freedom. P – significance.

EROD	**Origin**	Minho Lima	0.070 ± 0.006 0.024 ± 0.006	$F_{(1, 36)} = 27.961$ $P \leq 0.05$	**a** **b**
	Treatment	0 MP 3.1 6.3 12.5 25.0 50.0	0.032 ± 0.011 0.025 ± 0.011 0.038 ± 0.011 0.064 ± 0.011 0.060 ± 0.011 0.053 ± 0.011 0.057 ± 0.015	$F_{(5, 36)} = 1.993$ $P > 0.05$	No significant differences
	Interaction	Origin Treatment		$F_{(5, 36)} = 2.521$ $P > 0.05$	No significant differences
LPO	**Origin**	Minho Lima	5.429 ± 2.541 1.485 ± 1.873	$F_{(1, 87)} = 1.561$ $P > 0.05$	No significant differences
	Treatment	0 MP 3.1 6.3 12.5 25.0 50.0	1.836 ± 3.204 1.648 ± 3.303 14.993 ± 3.204 3.008 ± 3.204 0.917 ± 3.204 0.764 ± 3.158 1.034 ± 7.599	$F_{(5, 87)} = 2.458$ $P \leq 0.05$	Significant diferences
	Interaction	Origin Treatment		$F_{(5, 87)} = 2.599$ $P > 0.05$	No Significant differences

Conclusion and Future prespectives

The results of this study indicate that:

1) nickel has the potential to cause similar toxic effects on *Pomatoschistus microps* juveniles from both Minho and Lima river estuaries, with high levels of inhibition in the capability of fish to chase and ingest preys, reducing the predatory efficiency, rejecting the first hypothesis of juveniles from estuaries with different backgrounds of environmental contamination have different susceptibility to nickel;

2) microplastics present a "temporary protecting from death" effect on fish by delaying the effects of nickel and to attenuate them in a post exposure situation, validating the second hypothesis of the presence of microplastics in water influence the toxicity of nickel towards *Pomatoschistus microps* juveniles.

In the future it would be interesting to perform the same experience with fish from the same season, in order to make a better evaluation of the microplastics influence in the organisms besides the interaction with other contaminants, like nickel. Regarding one of the contaminants in the study be a heavy metal, it would be relevant to add to the biomarkers battery the determination of the metallothionein concentration, stress proteins known to be induced by essential and non-essential metals.

References

Almeida, C.M.R.; Mucha, A.P.; Vasconcelos, M.T.; 2011. Role of different salt marsh plants on metal retention in an urban estuary (Lima estuary, NW Portugal). *Estuarine, Coastal and Shelf Science,* **91**:243-249.

Andrady, A.L.; 2011. Microplastics in the marine environment. *Marine Pollution Bulletin*, **62**:1596-1605.

Atchison, G.J.; Henry, M.G.; Sandheinrich, M.B.; 1987. Effects of metals on fish behavior: a review. *Environmental Biology of fishes*, **18**:11-25.

Azevedo, I.; Ramos, S.; Mucha, A.P.; Bordalo, A.A.; 2013. Applicability of ecological assessment tools for management decision making: A case study from the Lima estuary (NW Portugal). *Ocean & Coastal Management*, **72**:54-63.

Beketov, M.A.; Liess, M.; 2012. Ecotoxicology and macroecology - Time for integration. *Environmental Pollution*, **162**:247-54.

Berrebi, P.; Rodriguez, P.; Tomasini, J.; Cattaneo-Berrebi, G.; Crivelli, A.J.; 2005. Differential distribution of the two cryptic species, *Pomatoschistus microps* and *P. marmoratus,* in the lagoons of southern France, with an emphasis on the genetic organisation of *P. microps*. *Estuarine, Coastal and Shelf Science*, **65**:708-716.

Bouchereau, J.-L.; Guelorget, O.; 1997. Comparison of three Gobiidae (Teleostei) life history strategies over their geographical range. *Oceanologica Acta*, vol **21**, nº 3.

Bradford, M.M.; 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**:248-254.

Burke, M.D.; Mayer, R.T.; 1974. Ethoxyresorufin: direct fluorometric assay of microsomal O-dealkylation which is preferentially inducible by 3-methylcholantrebe. *Drug Metabolism and Disposition*, **2**:583-388.

Casarett, L.J.; Klassen, C.D.; 2008. Toxic effects of metals in: Casaret and Doull's toxicology: the basic science of poisons. Seventh edition, McGraw-Hill Medical, pp 931, 950, 951.

Chapman, P.M.; 2002. Integrating toxicology and ecology: putting the "eco" into ecotoxicology. *Marine Pollution Bulletin*, **44**:7-15.

Chau, Y.K.; Kulikovsky-Cordeiro, O.T.R.; 1995. Occurrence of nickel in the Canadian environment. *Environmental Reviews*, **3**:95-120.

Claessens, M.; Cauwenberghe, L.V.; Vandegehuchte, M.B.; Janssen, C.R.; 2013. New techniques for the detection of microplastics in sediments and field collected organisms. *Marine Pollution Bulletin*, **7**:227-233.

Cole, M.; Lindeque, P.; Halsband, C.; Galloway, T.S.; 2011. Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin*, **62**:2588-2597.

Cole, M.; Webb, H.; Lindeque, P.K.; Fileman, E.S.; Haslband, C.; Galloway, T.S.; 2014. Isolation of microplastics in biota-rich seawater samples and marine organisms. *Scientific Reports*, **4**: 4528. DOI: 10.1038/srep04528.

Costa-Dias, S.; Sousa, R.; Antunes, C.; 2010a. Ecological quality assessment of the lower Lima Estuary. *Marine Pollution Bulletin*, **61**:234-239.

Costa-Dias, S.; Freitas, V.; Sousa, R.; Antunes, C.; 2010b. Factors influencing epibenthic assemblages in the Minho Estuary (NW Iberian Peninsula). *Marine Pollution Bulletin*, **61**:240-246.

Collignon, A.; Hecq, J.-H.; Galgani, F.; Collard, F.; Goffart, A.; 2013. Annual variation in neustonic micro-and meso-plastic particles and zooplankton in the Bay of Calvi (Mediterranean-Corsica). *Marine Pollution Bulletin*, Article in Press.

Denkhaus, E.; Salnikow, K.; 2002. Nickel essentiality, toxicity, and carcinogenicity. *Critical Reviews in Oncology/Hematology*, **42**:35-56.

Depledge, M.H.; Galgani, F.; Panti, C.; Caliani, I.; Casini, S.; Fossi, M.C.; 2013. Plastic litter in the Sea. *Marine Environmental Research*, **92**:279-281.

DerVartanian, D.V.; Chenoweth, M.R.; 2000. RAPID AND ACCURATE COLORIMETRIC DETERMINATION OF NICKEL AND COBALT IN PROTEIN SOLUTIONS. *United States Patent*.

Dolbeth, M.; Martinho, F.; Leitão, R.; Cabral, H.; Pardal, M.A.; 2007. Strategies of *Pomatoschistus minutus* and *Pomatoschistus microps* to cope with environment instability. *Estuarine, Coastal and Shelf Sciences*, **74**:263-273

Dolbeth, M.; Martinh, F.; Viegas, I.; Cabral, H.; Pardal, M.A.; 2008. Estuarine production of resident and nursery fish species: Conditioning by drought events? *Estuarine, Coastal and Shelf Science*, **78**:51-60.

Eisler, R.; 1998. Nickel hazards to fish, wildlife, and invertebrates: a synoptic review. *US Geological Survey, Biological Resources Division, Biological Science Report* USGS/BRD/BSR-1998-0001.

Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M.; 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, **7**:88-95.

Fendall, L.S.; Sewell, M.A.; 2009. Contributing to marine pollution by washing your face: Microplastics in facial cleansers. *Marine Pollution Bulletin*, **58**:1225-1228.

Ferreira, J.G.; Simas, T.; Nobre, A.; Silva, M.C.; Shifferegger, K.; Lencart-Silva, J.; 2003. Identification of Sensitive Areas and Vulnerable Zones in Transitional and Coastal Portuguese Systems-Application of the United States National Estuarine Eutrophication Assessment to the Minho, Lima, Douro, Ria de Aveiro, Mondego, Tagus, Sado, Mira, Ria Formosa and Guadiana Systems. *Instituto da Água and Institute of Marine Research,* Portugal.

Fonseca, V.F.; França, S.; Serafim, A.; Company, R.; Lopes, B.; Bebianno, M.J.; Cabral, H.N.; 2011a. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax, Sole senegalensis* and *Pomatoschistus microps*. *Aquatic Toxicology*, **102**:26-227.

Fonseca, V.F.; França, S.; Vasconselos, R.P.; Serafim, A.; Company, R.; Lopes, B.; 2011b. Short-term variability of multiple biomarker response in fish from estuaries: Influence of envvironmental dynamics. *Marine Environmental Research*, **71**:172-178.

Forget, J.; Pavillon, J.F.; Beliaeff, B.; Bocquene, G.; 1999. Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepoda, Harpacticoisa). *Environmental Toxicology and Chemistry*, **18**:912-918.

Fossi, M.C.; Panti, C.; Guerranti, C.; Coppola, D.; Giannetti, M.; Marsili, L.; Minutoli, R.; 2012. Are baleen whales exposed to the threat of microplastics? A case study of the Mediterranean fin whale (*Balaenoptera physalus*). *Marine Pollution Bulletin*, **64**:2374-2379.

Frasco, M.F.; Guilhermino, L.; 2002. Effects of dimethoate and bet-naphthoflavone on selected biomarkers of *Poecilia reticulata*. *Fish Physiology and Biochemistry*, **26**:149-156.

Frias, J.P.G.L.; Sobral, P.; Ferreira, A.M.; 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin*, **60**:1988-1992.

Garcia, L.M.; Castro, B.; Ribeiro, R.; Guilhermino, L.; 2000. Characterization of cholinesterases from guppy (*Poecilia reticulata*) muscle and it's in vitro inhibition by environmental contaminants. *Biomarkers*, **5**:274-284.

GEF, 2012. Impacts of Marine Debris on Biodiversity: Current Status and Potentional Solutions. GEF/STAP/C.43/Inf.04

George, S.G.; 1994. Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. In: Malins, D.C., Ostrander, G.H., (Eds.), Aquatic toxicology; Molecular, Biochemical and Cellular perspectives. Lewis Publishers, CRC press, pp. 37-85.

Gikas, P.; 2008. Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: A review. *Journal of Hazardous Materials*, **159**:187-203.

Gill, T.S.; Tewari, H.; Pande, J.; 1990. Use of fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. *Comparative Biochemistry and Physiology*, **97**C:287-292.

Gravato, C.; Guimarães, L.; Santos, J.; Faria, M.; Alves, A.; 2010. Comparative study about the effects of pollution on glass and yellow eels (*Anguilla anguilla*) from the estuaries of Minho, Lima and Douro Rivers (NW Portugal). *Ecotoxicology and Environmental Safety*, **73**:524-533.

Guilhermino, L.; Lopes, M.C.; Carvalho, A.P.; Soares, A.; 1996. Acetyrlcholisterase activity in juveniles of *Daphnia magna Straus. Bulletin of Environmental Contamination and Toxicology,* **57**:979-985.

Habig, W.H.; Pabst, M.J.; Jakoby, W.B.; 1974. Glutathione.S.Transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry,* **249**:7130-7139.

Hernández-Moreno, D.; Pérez-López, M.; Soler, F.; Gravato, C.; Guilhermino, L.; 2011. Effects of carbofuran on the sea bass (*Dicentrarchus labrax* L.): Study of biomarkers and behavior alterations. *Ecotoxicology and Environmental Safety*, **74**: 1905-1912.

Ivar do Sul, J.A.; Costa, M.F.; Barlett, M.; Cysneiros, F.J.A.; 2013. Pelagic microplastics around an archipelago of the Equatorial Atlantic. *Marine Pollution Bulletin*, **75**:305-309.

Ivar do Sul, J.A.; Costa, M.F.; 2014. The present and future of microplastic pollution in the marine environment. *Environment Pollution*, **185**:352-364.

Jackson, A.C.; Rundle, S.D.; 2008. Diet-shifts by an estuarine goby (*Pomatoschistus microps*) in the face of variable prey availability. *Journal of Experimental Marine Biology*, **361**:1-7.

Jackson, N.L.; 2013. 10.12 Estuaries. *Reference Module in Earth Systems and Environmental Sciences*, *Treatise on Geomorphology*, **10**: 308-327.

Jiang, J.-L.; Wang, G.-Z.; Mao, M.-G.; Wang, K.-J.; Li, S.-J; Zeng, C.-S; 2013. Differential gene expression profile of the calanoid copepod, *Pseudodiaptomus annandalei*, in response to nickel exposure. *Comparative Biochemistry and Physiology, Part C*, **157**:203-211.

Kozlovskaya, V.I.; Mayer, F.I.; Menzikova, O.V.; Chuyko, G.M.; 1993. Cholinesterase of aquatic animals. *Reviews of Environmental Contamination and Toxicology*, **132**:117-142.

Kubrak, O.I.; Husak, V.V.; Rovenko, B.M.; Poigner, H.; Mazepa, M.A.; Kriews, M.; Abele, D.; Lushchak, V.I.; 2012. Tissue specificity in nickel uptake and induction of oxidative stress in the kidney and spleen of goldfish *Carassius auratus*, exposed to waterborne nickel. *Aquatic Toxicology*, **118-119**:88-96.

Kubrak, O.I.; Husak, V.V.; Rovenko, B.M.; Poigner, H.; Kriews, M.; Abele, D.; Lushchak, V.I.; 2013. Antioxidant system efficiently protects goldfish from Ni^{2+} induced oxidative stress. *Chemosphere*, **90**:971-976.

Laur, K.; Ojaveer, H.; Simm, M.; Klais, R.; 2014. Multidecadal dynamics of larval gobies *Pomaroschistus spp*. in response to environmental variability in a shallow temperate bay. *Estuarine, Coastal and Shelf Science*, **136**:112-118.

Leitão, R.; Martinho, F.; Neto, J.M.; Cabral, H.; Marques, J.C.; Pardal, M.A.; 2006. Feeding ecology, population structure and distribution of *Pomatoschistus microps* (KØyer, 1838) and *Pomatoschistus minutus* (Pallas, 1770) in a temperate estuary, Portugal. *Estuarine, Coastal and Shelf Science*, **66**:231-239.

Little, E.E.; Finger, S.E.; 1990. Swimming behavior as an indicator of sublethal toxicity in fish. *Environmental Toxicology and Chemistry*, **9**:13-19.

Lushchak, V.I.; 2008. Oxidative stress as a component of transition metal toxicity in fish. In: Svensson, E.P.(Ed.). Aquatic Toxicology Research Focus. Nova Science Publishers Inc, Hauppauge, NY. pp 1-29.

Lusher, A.L.; McHugh, M.; Thompson, R.C.; 2013. Ocurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine Pollution Bulletin*, **67**:94-99.

Magalhães, D.P.; Filho, A.S.F.; 2008. A Ecotoxicologia como ferramenta no biomonitoramento de ecossistemas aquáticos. *Oecol. Bras.*, **12**(3):355-381.

Maretec (Marine Environment & Tecnology Center), n.d. Definição do Limite de Jusante dos Estuários Portugueses. Descrição – Estuário Minho.

In:<http://www.maretec.mohid.com/Estuarios/MenusEstuarios/Descri%C3%A7%C3%A3o/descricao_Minho.htm>. Access: 15/09/2014.

Martins, J.; Sobral, P.; 2011. Plastic marine debris on the Portuguese coastline: A matter of size?. *Marine Pollution Bulletin*, **62**:2649-2653.

McCarthy, J.F.; Shugart, L.R.; 1990. Biomarkers of Environmental Contamination. Boca Raton, FL: Lewis Publishers.

Mohammed, E.H.; 2014. Biochemical Response og th Cyclopoida Copepod *Apocyclops borneonsis* Exposed to Nickel. *Jordan Journal of Biological Sciences*, **7**:41-47.

Monteiro, M.; Quintaneiro, C.; Morgado, F.; Soares, A.M.V.M.; Guilhermino, L.; 2005. Characterization of the cholinesterases present in head tissue of the estuarine fish *Pomatoschistus microps*: Application to biomonitoring. *Ecotoxicology and Environmental Safety*, **62**:341-347.

Monteiro, M.; Quintaneiro, C.; Pastorinho, M.; Pereira, M.L.; Morgado, F.; Guilhermino, L.; Soares, A.M.M.; 2006. Acute effects of 3,4-dichloroaniline on biomarkers and spleen histology of the common goby *Pomatoschistus microps*. *Chemosphere,* **62**:1333-1339.

Monteiro, M.; Quintaneiro, C.; Nogueira, A.J.A.; Morgado, F.; Soares, A.M.V.M.; Guilhermino, L.; 2007. Impact of chemical exposure on the fish *Pomatoschistus microps* KrØyer (1838) in estuaries of the Portuguese Northwest coast. *Chemosphere,* **66**:514-522.

Moore, C.J.; 2008. Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. *Environ. Res*., **108**:131-139.

Nyitrai, D.; Martinho, F.; Dolbeth, M.; Rito, J.; Pardal, M.A.; 2013. Effects of local and large-scale climate patterns on estuarine resident fishes: The example of *Pomatoschistus microps* and *Pomatoschistus minutus*. *Estuarine, Coastal and Shelf Science,* **135**:260-268.

OECD, 1992. Test No.203: Fish, Acute Toxicity Test.

OECD, 2012. New and Emerging Water Pollutants arising from Agriculture. Background report in Water Quality and Agriculture: Meeting the Policy Challenge. http://www.oecd.org/tad/sustainable-agriculture/49848768.pdf

Ohkawa, H.; Ohishi, N.; Yagi, K.; 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**:351-358.

Oliva, M.; Perales, J.A.; Gravato, C.; Guilhermino, L.; Galindo-Riaño, M.D.; 2012. Biomarkers response in muscle of Senegal sole (*Solea senegalensis*) from a heavy metals and PAHs polluted estuary. *Marine Pollution Bulletin*, **64**:2097-2108.

Oliveira, M.; Gravato, C.; Guilhermino, L.; 2012. Acute toxic effects of pyrene on *Pomatoschistus microps* (Teleostei, Gobiidae): Mortality, biomarkers and swimming performance. *Ecological Indicators*, **19**:206-214.

Oliveira, M.; Ribeiro, A.; Hylland, K.; Guilhermino, L.; 2013. Single and combined effects of microplastics and pyrene on juveniles (0+group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecological Indicators,* **34**:641-647.

Osswald, J.; Carvalho, A.P.; Guimarães, L.; Guilhermino, L.; 2013. Toxic effects of pure anatoxin-a on biomarkers of rainbow trout, *Onchorynchus mykiss*. *Toxicon*, **70**:162-169.

Pampoulie, C.; 2001. Demographic structure and life history traits of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae) in a Mediterranean coastal lagoon (Rhône River delta, France). *Acta Oecol*, **22**:253-357.

Pane, E.F.; Richards, J.G.; Wood, C.M.; 2003. Acute waterborne nickel toxicity in the rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. *Aquatic Toxicology*, **63**:65-82.

Payne, J.F.; Fancey, L.L.; Rahimtula, A.D.; Porter, E.L.; 1987. Review and perspevtive on the use of mixed-function oxygenase enzymes in biological monitoring. *Comparative Biochemistry and Physioogy.,* **86**C: 233-245.

Pinto, R.; Martins, F.C.; 2013. The Portuguese National Strategy for Integrated Coastal Zone Management as a spatial planning instrument to climate change adaptation in the Minho River Estuary (Portugal NW-Coastal Zone). *Environmental Science & Policy*, **33**:76-96.

Peakall, D.; 1992. Animal biomarkers as pollution indicators, first ed. Chapman & Hall, London.

Peakall, D.B.; Walker, C.H.; 1994. The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology*, **3**:173-179.

Plastics Europe, 2013. Plastics - the Facts 2013 An analysis of European Latest plastics production, demand and waste data for 2012 (14.10.2013). http://www.plasticseurope.org/Document/plastics-the-facts-2013.aspx?Page=DOCUMENT&FolID=2

Pourkhabbaz, A.; Khazaei, T.; Behravesh, S.; Ebrahimpour, M.; Pourkhabbaz, H.; 2011. Effects of water hardness on the toxicity of cobalt and nickel to a freshwater fish, *Capoeta fusca. Biomed Environ Sci*, **24**(6):656-660.

Pockberger, M.; Kellnreitner, F.; Ahnelt, H.; Asmus, R.; Asmus, H.; 2014. An abundant small sized fish as keystone species? The effect of *Pomatoschistus microps* on food webs and its trophic role in two intertidal benthic communities: A modeling approach. *Journal of Sea Research*, **86**:86-96

Pritchard, D.W.; 1967. What is an estuary: a physical viewpoint. *American Association for the Advancement of Science*, **83**:3-5.

Quintaneiro, C.; Querido, D.; Monteiro, M.; Guilhermino, L.; Morgado, F.; Soares, A.M.V.M.; 2008. Transport and acclimation conditions for the use of an estuarine fish (*Pomatoschistus microps*) in ecotoxicity bioassays: Effects on enzymatic biomarkers. *Chemosphere*, **71**:1803-1808.

Ramos, S.; Ré, P.; Bordalo, A.A.; 2010. Recruitment of flatfish species to an estuarine nursery habitat (Lima estuary, NW Iberian Peninsula). *Journal of Sea Research,* **64**:473-486.

Sáenz, L.A.; Seibert, E.L.; Zanette, J.; Fiedler, H.D.; Curtius, A.J.; Ferreira, J.F.; Almeida, E.A.; Marques, M.R.F.; Bainy, A.C.D:; 2010. Biochemical biomarkers and metals in *Perna perna* mussels from mariculture zones of Santa Catarina, Brazil, *Ecotoxicology and Environmental Safety,* **73**:796-804.

Sanchez, W.; Porcher, J.-M.; 2009. Fish biomarkers for environmental monitoring within the Water Framework Directive of the Europe Union. *Trends in Analytical Chemistry*, **28**:2.

Sanchez, W.; Bender, C.; Porcher, J.-M.; 2014. Wild gudgeons (*Gobio gobio*) from French rivers are contaminated by microplastics: Preliminary study and first evidence. *Environmental Research*, **128**:98-100.

Scott, G.R.; Sloman, K.A.; 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquatic toxicology*, **68**:369-392.

Serafim, A.; Company, R.; Lopes, B.; Fonseca, V.F.; França, S.; Vasconcelos, R.P.; 2012. Application of an integrated biomarker response index (IBR) to assess temporal variation of environmental quality in two Portuguese aquatic systems. *Ecological Indicators*, **19**:215-225.

Setälä, O.; Fleming-Lehtinen, V.; Lehtiniemi, M.; 2014. Ingestion and trasfer of microplastics in the planktonic food web. *Environmental Pollution*, **185**:77-83.

Shah, A.A.; Hasan, F.; Hameed, A.; Ahmed, S.; 2008. Biological degradation of plastics: A comprehensive review. *Biotechnology Advances*, **26**:246-265.

Sivan, A.; 2011. New perspectives in plastic biodegradation. *Current Opinion in Biotechnology*, **22**:422-426.

Svecevicius, G.;2010. Acute Toxicity of Nickel to Five Species of Freshwater Fish. *Polish Journal of Environmental Studies*, **19**:453-456.

Truhaut, R.; 1977. Ecotoxicology: objectives, principles and perspectives. *Ecotoxicology and Environmental Safety*, **1**:151-173.

Van der Oos, R.; Beyer, J.; Vermeulen, N.P.E.; 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, **13**:57-149.

Vieira, L.R.; Sousa, A.; Frasco, M.F.; Lima, I.; Morgado, F.; Gruilhermino, L.; 2008. Acute effects of Benzo[a]pyrene, anthracene and a fuel oil on biomarkers of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Science of the total environment*, **395**:87-100.

Vieira, L.R.; Gravato, C.; Soares, A.M.V.M.; Morgado, F.; Guilhermino, L.; 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarkers to behaviour. *Chemosphere*, **76**:1416-1427.

Wang, W.X.; & Wang, G.Z.; 2010. Oxidative damage effects in the copepod *Tigriopus japonicas* Mori experimentally exposed to nickel. *Ecotoxicology*, **19**: 273-284.

Williams, P.L.; James, R.C.; Roberts, S.M.; 2000. Properties and effects of metals in: General principles of toxicology. In: *Principles of Toxicology: Environmental and industrial applications*. Second edition, John Wiley & Sons, Inc., pp 3-5/326-342.

Wright, S.L.; Thompson, R.C.; Galloway, T.S.; 2013. The physical impacts of microplastics on marine organisms: A review. *Environment Pollution*, **178**:483-492.

Zar, J.H.; 1999. Biostatistical Analysis. Prentice Hall, New Jersey.